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CHARACTERIZATION OF THE SOIL GLOBIFORME BACTERIA

by

Haskell C. Phillips

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Soil Bacteriology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

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Dean of Graduate College

Iowa State College
1951

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INTRODUCTION

Much of the bacteriology of the soil has been concerned with the biological transformations of mineral nutrient materials in the presence of the total soil microflora. Studies of individual soil microorganisms have been confined largely to those bacteria that are known to effect nitrogen fixation, nitrification or denitrification, or decomposition of specific substrates, such as, for example, cellulose, or else to those bacteria that possess striking morphological or cultural characteristics, even though such bacteria may form only a very minor fraction of the total soil population. Studies of the dominant microorganisms in soils have been undertaken by relatively few workers. Such workers, although from widely scattered geographical locations, uniformly have noted that small, slow-growing, pleomorphic bacteria, relatively inert in their cultural responses, are the most numerous types in soil. The exact function of these bacteria in the soil remains obscure. Their cultural characteristics, other than that they lack distinctive physiological properties, likewise are poorly defined. It is not surprising that their taxonomic position is at present controversial. In recent years, this group of microorganisms has been designated rather vaguely as soil globiforme bacteria, as soil corynebacteria or, less commonly, as soil diphtheroids.

In current taxonomic systems for bacteria, the soil globiforme bacteria are designated by such generic names as Arthrobacter, Corynebacterium, and Jensenia. Obviously, if the proponents of each of these names are dealing with the same microorganisms, no more than one of the

names can be recognized as valid. If on the other hand the soil globiforme bacteria are sufficiently heterogeneous that they do belong in separate genera, the relationships of these genera to each other and to existing genera such as Cellulomonas and Microbacterium are poorly defined. The confusion at the generic level is paralled by that at the specific level.

It has been the purpose of the present investigation to characterize the morphology and physiology of the globiforme bacteria predominant in soil. Emphasis has been given to the study of bacterial cultures previously named by other workers in order to determine whether identical organisms currently are known under different names and in an effort to establish criteria that will serve to define the generic and specific relationships of the soil globiforme bacteria.

REVIEW OF LITERATURE

The difficulty of separating corynebacteria of animal origin from less differentiated bacteria from other sources was apparent to Lehmann and Neumann (38) and to many succeeding writers. It became recognized that saprophytic corynebacteria occurred in soil, air, and water. These forms, considered clinically unimportant, received very little attention from medical bacteriologists. For the most part, soil bacteriologists gave their attention to bacteria accomplishing mineral nutrient transformations believed important in soil productivity. Conn (16, 17), however, directed his efforts for a number of years to a study of the general soil microflora, and he became particularly interested in the pleomorphic, gram-variable microorganisms in soil. In 1928, he (18) described the species Bacterium globiforme as representative of this group of commonly occurring soil bacteria. This species subsequently was designated Achromobacter globiforme by Bergey et al. (5). Bacteria undoubtedly identical with the globiforme group of Conn have been considered as belonging to Mycobacterium by Krassilnikov (35), and to Corynebacterium, by Jensen (31), Topping (56), Clark (12), and Lochhead (40). The work of Jensen (31) played an especially dominant part in the development of a trend to broaden the genus Corynebacterium to include therein a number of bacteria of soil and plant origin.

During the course of microbiological analyses of Australian soils, Jensen (30) noted the remarkably frequent occurrence of small angular rods. Following careful study of the morphology and physiology of

these organisms, Jensen (31) described several new species from soil as members of the genus Corynebacterium. His new species were Corynebacterium simplex, C. tumescens, and C. filamentosum. In addition, he established new combinations in Corynebacterium to accomodate seven species previously assigned to four other genera. The species transferred to Corynebacterium were the following: Aplanobacter insidiosum McCulloch, A. michiganense Erwin Smith; Bacterium helvolum (Zimmermann) Lehmann and Neumann, B. nubilum (Frankland) Lehmann and Neumann; Cellulomonas fima (McBeth and Scales) Bergey et al; Microbacterium lacticum Orla-Jensen, and M. liquefaciens Orla-Jensen. These several new combinations were made by Jensen because of his firm belief that the bacteria concerned formed a natural and consistent group.

Plant pathologists showed a willingness to follow the suggestion of Jensen (31) that certain phytopathogenic bacteria should be designated as corynebacteria. Skaptason and Burkholder (50) transferred Bacterium sepedonicum Spiekermann and Kotthoff, the organism causing bacterial ring rot of potatoes, to Corynebacterium. Dowson (26) made new combinations in Corynebacterium to accommodate Aplanobacter rathayi Erwin Smith, Bacterium flaccumfaciens Hedges, and Phytomonas fasciens Tilford. Starr and Pirone (53) described the new species Corynebacterium poinsettiae as the cause of a disease of the common ornamental poinsettia. Breed et al. (11) recognized these several new combinations or species, and, in addition, made new combinations to place in Corynebacterium the following: Aplanobacter agropyri O'Gara,

Pseudomonas hypertrophicans Stahel, and Pseudomonas tritici Hutchinson.

In addition to their recognition of phytopathogenic corynebacteria and of several saprophytic corynebacteria from soil as members of the genus Corynebacterium, Breed et al. (11) recognized the transfer by Jensen (31) of a cellulolytic bacterium to Corynebacterium from Cellulomonas.

The genus Cellulomonas was established by Bergey et al. (3) principally on the character of cellulolytic activity and primarily for some 20 species of cellulolytic bacteria initially described by Kellerman and associates (32, 33, 42, 43). The genus included such diverse types as polar flagellates which were later transferred to Pseudomonas, gram-variable nonmotile rods later placed in Corynebacterium gram-negative nonmotile bacteria later returned to Bacterium, and peritrichous, non-sporeforming bacteria which Breed, et al. (11) retained in Cellulomonas, at the same time commenting that the name was unsuitable. The genus Cellulomonas was not inserted in the outline classification used in the sixth edition of Bergey's Manual (11), but instead was placed as an appendix to Bacterium.

Jensen (31) observed that Cellulomonas fima showed plainly the morphology of the corynebacteria, and accordingly, he transferred the species to Corynebacterium. He did not at the same time have available for study additional cultures labelled Cellulomonas. Subsequently, Clark (13) obtained stock cultures of several species of Cellulomonas, and found them to show no generic distinction from Corynebacterium fimi. The question thus arises whether or not these additional cellulolytic

bacteria are also to be grouped generically with the soil globiforme bacteria of Conn (18) and Jensen (31).

Much of the confusion concerning the assignment of bacteria of soil and plant origin to the genus Corynebacterium is centered on the uncertain position of this genus in general schemes of bacterial taxonomy. Historically, the genus Corynebacterium was established by Lehmann and Neumann (36) in 1896, with the type species C. diphtheriae, the already well known "Klebs Loeffler bacillus". The genus, together with Mycobacterium and Oospora, was placed by its authors in the Hyphomycetes, their fourth major grouping of microorganisms. In a second edition by Lehmann and Neumann (37), Oospora was re-designated Actinomyces, and the Hyphomycetes, as the Actinomycetes. In the seventh edition (39), the two genera, Corynebacterium and Mycobacterium comprised the family Proactinomycetaceae in the order Actinomycetales.

The extent to which the schemes of other taxonomists, during the period 1896 to 1927, deviated from the proposals of Lehmann and Neumann has been adequately summarized in the introductory pages of the sixth edition of Bergey's Manual (11). For the most part, Corynebacterium and Mycobacterium were placed within the same family, and usually in the order Actinomycetales. There were relatively few expressions of disagreement with such a system. Notably, Winslow et al. (60) recognized six genera in the family Mycobacteriaceae in the order Eubacteriales. The genera so grouped were the following: Corynebacterium, Mycobacterium, Nocardia, Fusiformis, Actinomyces, and Leptotrichia. Their proposed placement of these genera under Eubacteriales was not

generally accepted in the years immediately following publication.

In agreement with the outline classification of Lehmann and Neumann (39), Bergey et al. (3) placed the family Mycobacteriaceae, including Corynebacterium and Mycobacterium, in the order Actinomycetales. Four succeeding editions of Bergey's Manual (4, 5, 6, 7) retained this placement of the genus Corynebacterium. Nor was the taxonomic treatment accorded to the genus by Pringsheim (47), Janke (29), or Pribram (46) greatly different.

Recently, and concurrently with the trend to broaden Corynebacterium to include many bacteria of soil and plant origin, there again appeared some suggestions that the genus Corynebacterium should be assigned to the order Eubacterialess and not to the Actinomycetales. Kluyver and Van Niel (34) recognised the Mycobacteriaceae as a family of permanently nonmotile, rod-shaped bacteria, and proposed to include therein two tribes, the Corynebacterieae and the Mycobacterieae. Stanier and Van Niel (52) also included both Mycobacterium and Corynebacterium in the Eubacteriales. In criticism of Bergey's Manual, they (52) stated: "The inclusion of the mycobacteria and corynebacteria (in Actinomycetales) leads to confusion, since these forms can so readily be taken for representatives of Eubacteriales." On the other hand, they admitted that the dividing line between the genera Mycobacterium and Proactinomyces (i.e., Nocardia) was a tenuous one.

Although Stanier and Van Niel (52) had included both the mycobacteria and corynebacteria in Eubacteriales, Breed et al. (11) separated the two genera, placing Corynebacterium in the order Eubacteriales

and the genus Mycobacterium in the Actinomycetales.

Several workers have raised objection to any such assignment and separation of the corynebacteria and mycobacteria. Conn and Dimmick (21) regarded the two genera as closely related and as members of the same order. Bisset and Moore (9) expressed the opinion that the mycobacteria and corynebacteria possess a morphology strikingly different from that of the true bacteria. They grouped Mycobacterium, Corynebacterium, and Nocardia in the family Mycobacteriaceae, and the genus Actinomyces and their newly proposed genus Jensenia in the family Actinomycetaceae, with both families in the order Actinomycetales. Cowan (24) also placed Mycobacterium and Corynebacterium in the family Mycobacteriaceae, but he proposed that this family be placed in a separate order, Mycobacteriales. The families Actinomycetaceae and Propionibacteriaceae were placed in the order Actinomycetales. Altogether, it appears that the great majority of those who have worked or are working intensively with the corynebacteria oppose any wide separation of the mycobacteria and the corynebacteria.

Two recent expressions of opposition to assignment of soil and plant corynebacteria to the genus whose type is C. diphtheriae have been accompanied by proposals for new generic names for the soil globiforme or diphtheroid bacteria.

Conn and Dimmick (21) objected to the inclusion of an ever greater variety of organisms in Corynebacterium. They undertook comparative study of animal, plant, and soil diphtheroids and concluded that the three groups were sufficiently dissimilar, especially in physiology,

to preclude their classification in a single genus. They proposed that the soil diphtheroids be placed in a genus Arthrobacter. This generic term was first proposed in 1895 by Fischer (28) to cover the nonflagellate, rod-shaped bacteria producing "arthrospores". No type was described, and the genus can not be considered to have been validly published prior to the year 1947 (21).

Arthrobacter was distinguished from Corynebacterium partly on morphological and partly on physiological differences. Members of Corynebacterium were unable to grow on synthetic media or to liquefy gelatin, and generally showed gram-negative cells in old rather than in young cultures. Members of Arthrobacter showed greater irregularity in cell morphology, particularly in branching and in production of larger coccoid bodies. Arthrobacter cultures grew readily on synthetic media containing inorganic nitrogen. They liquefied gelatin and digested milk, but showed only weak production of acid from any sugar. Conn and Dimmick (21) initially described three species of Arthrobacter, namely, A. globiforme, A. helvolum, and A. tumescens. Subsequently, the same authors (22) described A. simplum as an additional species. It differed from A. globiforme only in its lack of diastatic action on starch. Recently, Conn (19) has suggested that the yellow chromogenic forms of A. globiforme be designated as A. aurescens. Lochhead (41) found that yellow-pigmented soil diphtheroids were especially abundant in the rhizospheres of plants. No chromogenic cultures of the globiforme group were obtained from uncropped soil, whereas 42.3 per cent of all strains of this group isolated from soil adhering to roots produced

yellow colonies.

Bisset and Moore (9) expressed the opinion that the mycobacteria and corynebacteria possess a morphology differing greatly from that of the true bacteria. They also believed that the soil corynebacteria were sufficiently dissimilar to the diphtheria organism to preclude their assignment of the same genus. Bisset and Moore relied almost entirely on the tannic acid-violet staining technique (48) for cell walls in making their taxonomic separations. They largely ignored the extended and very valuable earlier work of Conn, Jensen, and others. Soil diphtheroids were described as unicellular, and therefore distinct from the true corynebacteria, which were described as multicellular. Bisset and Moore (9) proposed that the soil diphtheroids be placed in a new genus Jensenia; no type species was described. After this omission was called to their attention, the genus was again proposed (10), this time with the type as Jensenia canicruria. This species was described as failing to ferment carbohydrates or to hydrolyze polysaccharides or proteins, and also, as failing to reduce nitrates. Inasmuch as Arthrobacter globiforme ordinarily is described as positive in all these respects, it appears probable that the two species are, in fact, separate entities.

The contention of Bisset and Moore (9) that the soil diphtheroids are distinct morphologically from true corynebacteria has not received published confirmation. Doubt on the adequacy of their proposed system is suggested by the authors themselves (9), who admitted that the greatest defect of the system is that the characters defining the various

groups are relative, in that they sometimes lie within the limits of variability of a single species at various ages and conditions of culture.

Neither Conn and Dimmick (21, 22) nor Bisset and Moore (9, 10) reported any occurrence of activity motility among the soil diphtheroids. Topping (56) encountered motility among the Gram-positive pleomorphic rods from soil, and cited an opinion of Ørskov that motility is by no means a rare property in the angular rods and small mycelial fungi. Topping believed it impossible to distinguish certain motile and non-motile pleomorphic organisms on any characteristic other than motility, and concluded that it seemed reasonable to classify both in a single group.

Taylor (54) and Lochhead (41) also reported the occurrence of motility in the Bacterium globiforme group. Dowson (26, 27) and others (11, 50, 53) have placed in Corynebacterium some phytopathogenic bacteria which show motility, and also others which fail to do so. Dowson (26) has noted that C. flaccumfaciens is reported as motile by American workers, whereas an Australian strain is reported as nonmotile. Discrepancy in the motility responses of C. michiganense was encountered by Conn, Wolfe, and Ford (23). Clark and Carr (14) have reported from one to several flagella both for cultures of Arthrobacter received from Conn and for cultures of certain cellulolytic corynebacteria previously described as nonmotile. Breed et al. (11) have stated that the reports of motile species in Corynebacterium present a puzzling taxonomic problem.

The majority of the investigations on soil globiforme bacteria have been concerned with their taxonomy. Knowledge concerning the functions or significance of the globiforme group in soil is limited. Conn (18) found that Bacterium globiforme was abundant in productive soils but apparently absent from soils of low fertility. Taylor and Lochhead (55) failed to find the abundance of the organism in soil to be correlated with soil productivity. Taylor (54) was able to isolate B. globiforme from garden, prairie, marsh, orchard, and field crop soils, whether acid, neutral, or alkaline in reaction, and from soils located on the rim of the Arctic Circle and on the Atlantic and Pacific coasts of southern Canada. Conn and Darrow (20) have suggested that inasmuch as the globiforme bacteria can use soluble nitrogen, they may serve to retain soil nitrogen that would otherwise be removed by drainage or utilized by plants. Taylor (54) planted sterilized seeds of alfalfa, red clover, and timothy in test tubes, and inoculated them with B. globiforme. Although the bacteria proliferated around the roots, the plants showed no beneficial or harmful effects from the presence of the bacteria.

In brief recapitulation, several summarizing statements are presented concerning the soil globiforme bacteria. The morphology and physiology of this group of bacteria remains poorly defined, and there is little agreement on the characteristics that may be used for their generic or specific identification. Strong critism of the trend to include the soil corynebacteria in the genus Corynebacterium has been expressed, and two separate proposals have been advanced for a new

generic designation. The genera Arthrobacter and Jensenia have been proposed. The validity of these proposals has not been fully examined, nor has the relationships of certain phytopathogenic and cellulolytic corynebacteria to the soil and animal diphtheroids been satisfactorily defined. Although the significance of the soil globiforme group is not yet known, their abundance in soil emphasizes the desirability of their more adequate characterization.

MATERIALS AND METHODS

Bacterial Cultures Employed

The experimental work undertaken in this study was based upon 55 cultures of bacteria initially isolated by workers and upon five cultures isolated directly from local soil. All cultures initially isolated or named by other workers were received from Dr. Francis E. Clark, Department of Agronomy, Iowa State College. The five cultures isolated locally were from the plow layer of a cultivated Clarion loam located on the Agronomy Farm, Ames, Iowa. By use of successive serial dilutions in sterile distilled water, soil suspensions of one part in one million were prepared. One milliliter of the prepared suspension was seeded in egg albumen agar (58). After ten days of incubation at 28° C., 50 individual colonies were picked for re-purification and microscopic examination. Five of these were found to show the pleomorphism and Gram-staining variability described by Conn (18) as characteristic of Bacterium globiforme, and they were retained for further study.

The 60 cultures employed in the current investigation are listed in Table 1. In order to facilitate the expression of experimental results, the 60 cultures were divided into 6 subgroups. These sub-groups are also shown in Table 1.

Table 1. Cultures Employed in Current Study

Culture No.	Name under which received	Strain designation	Information concerning initial isolation
Group I.	*Arthrobacter*:		
1.	*Arthrobacter aurescens*	none	Cultures 1 through 9 named and isolated by H. J. Conn and co-workers, Geneva, New York.
2.	*Arthrobacter aurescens*	DK-10	
3.	*Arthrobacter aurescens*	DK-22	
4.	*Arthrobacter globiforme*	none	
5.	*Arthrobacter globiforme*	ATCC-8010	
6.	*Arthrobacter simplum*	none	
7.	*Arthrobacter simplum*	DK-17	
8.	*Arthrobacter simplum*	DK-21	
9.	*Arthrobacter simplum*	LT-II	
10.	*Arthrobacter helvolum*	none	Cultures 10 through 13 isolated by H. L. Jensen in Australia.
11.	*Arthrobacter tumescens*	none	
12.	*Corynebacterium tumescens*	ATCC-6947	
13.	*Corynebacterium simplex*	ATCC-6946	
14.	*Bacterium globiforme*	none	Cultures 14 through 17 initially from A. G. Lochhead, Ottawa, Canada.
15.	*Bacterium globiforme*	none	
16.	*Bacterium globiforme*	none	
17.	*Bacterium globiforme*	none	
18.	Unnamed	22	Cultures 18 through 21 initially from L. Topping, Edinburgh, Scotland.
19.	Unnamed	81	
20.	Unnamed	159	
21.	Unnamed	178	
22.	Unnamed	—	Cultures 22 through 26 isolated by the writer at Ames, Iowa.
23.	Unnamed	—	
24.	Unnamed	—	
25.	Unnamed	—	
26.	Unnamed	—	

Table 1. (continued)

Culture No.	Name under which received	Strain designation	Information concerning initial isolation
Group II. *Jensenia-Nocardia*:			
27.	*Jensenia canicruria*	none	Bisset and Moore (9) Birmingham, England
28.	Unnamed	64	Cultures 28 through 30 initially from L. Topping Edinburgh, Scotland
29.	Unnamed	L-27	
30.	Unnamed	114	
31.	*Nocardia rubra*	none	N. M. McClung, Lawrence, Kansas.
Group III. Animal diphtheroids:			
32.	*Corynebacterium equi*	29	Cultures 32 through 35 initially from R. A. Packer, Ames, Iowa
33.	*Corynebacterium equi*	190	
34.	*Corynebacterium pyogenes*	16738H	
35.	*Corynebacterium renale*	16738NH	
36.	Unnamed	12B	Cultures 36 through 45 initially from E. V. Morse, Madison, Wisconsin.
37.	Unnamed	Bullmill	
38.	Unnamed	70P	
39.	Unnamed	70-0	
40.	Unnamed	B1	
41.	Unnamed	161Sh	
42.	Unnamed	152SH	
43.	Unnamed	1880	
44.	Unnamed	1807	
45.	Unnamed	1834	

Table 1. (continued)

Culture No.	Name under which received	Strain designation	Information concerning initial isolation
Group IV. Cellulolytic corynebacteria:			
46.	Cellulomonas fima	NRS-133	Cultures 46 through 54 initially isolated by Kellerman & co-workers Washington, D. C.
47.	Cellulomonas uda	NRS-136	
48.	Cellulomonas liquata	NRS-485	
49.	Cellulomonas biazotea	NRS-486	
50.	Cellulomonas cellasea	NRS-487	
51.	Cellulomonas flavigena	NRS-482	
52.	Cellulomonas gelida	NRS-488	
53.	Cellulomonas subalba	NRS-489	
54.	Cellulomonas perlurida	NRS-131	
Group V. Phytopathogenic corynebacteria:			
55.	Corynebacterium flaccumfaciens	ATCC-7392	P. A. Ark, Berkley, Cal.
56.	Corynebacterium michiganense	ATCC-10202	
57.	Corynebacterium poinsettiae	ATCC-9682	M. P. Starr, Davis, Cal.
Group VI. Microbacteria:			
58.	Microbacterium lacticum	none	F. E. Nelson, Ames, Iowa
59.	Microbacterium lacticum	OJ-6	R. N. Doetsch College Park, Md.
60.	M. Lacticum, var. liquefaciens	C-16	

Microscopy and Staining

Cultures were examined by means of the light microscope under the following conditions: (a) unstained; (b) stained by a tannic acid-violet method, and (c) stained by Gram's method.

Unstained cells from broth cultures were examined in liquid mounts for presence or absence of motility and for the occurrence of cell branching. Unstained cells were also examined *in situ* on the nutrient agar surfaces on which they had been grown in order to observe whether or not early mycelium formation occurred. To secure surface cultures on agar suitable for direct microscopy, the following procedure was employed: sterilized petri dishes each containing a standard microscope slide were filled with melted nutrient agar sufficient to cover the slide with a layer of agar 2 mm. deep. Solidified agar was inoculated lightly with a wire loop. After 12 to 18 hours, or as soon as barely visible growth developed, the glass slide with its covering of agar was transferred to a microscope stage for direct examination. Such agar mounts could be maintained for later examinations, after intervals ranging up to 16 hours, simply by returning the glass slides to moist petri dishes upon completion of the direct microscopy.

The majority of the named cultures in the collection were examined by means of a tannic acid and crystal violet technique recommended for staining cell walls (48). Cultures were grown for from 8 to 24 hours in nutrient broth. Cells were centrifuged for 15 minutes at 2,800 rpm, and the supernatent liquid discarded. Sedimented cells were re-suspended

in water, smeared on glass cover slips, and lightly heat fixed. The smears were then mordanted for 30 minutes in 5 per cent tannic acid in aqueous solution, washed, and stained for 25 seconds in 0.02 per cent aqueous crystal violet. After they were dried, cover slips were inverted and examined with an oil immersion objective.

Gram staining was performed according to Hucker's method (15). It was carried out on all cultures in the collection. For a given culture, cells were taken from separate nutrient agar slants, inoculated 14 hours, and 2, 5, and 10 days previously, and stained simultaneously on separate areas of a single glass slide. In addition, care was taken to standardize the several steps in the staining technique. Stained smears were examined with a 100x oil immersion objective and with paired 10 X oculars in a standard light microscope, using light from a microscope lamp fitted with a daylight filter.

Representative cultures in the collection were examined with an RCA electron microscope, model EMU. Cultures were grown for intervals ranging from 8 hours to 14 days on nutrient agar slants at 28° C. Surface growth washed from the slant was suspended in distilled water and centrifuged at 2500 rpm. for 5 minutes, then re-suspended in fresh distilled water for two additional washings. Washed cells were transferred to collodion covered screens for examination in a 50 kv electron beam. Observations were commonly made at magnifications of 6000 or 9000 X.

Certain cultures known to show motility were seeded in nutrient broth. After 18 to 26 hours, small portions of broth were transferred

directly to collodion screens and allowed to dry. These specimens were washed three times by applying and withdrawing drops of distilled water with the aid of a medicine dropper. The specimens were then shadow cast with gold in a vacuum chamber.

The electron microscopy and the electron photography were performed by Dr. P. H. Carr, Department of Physics, Iowa State College.

Media and Conditions for Observing General Growth Characteristics

Macroscopic appearance of growth on nutrient agar was observed for abundance, general appearance (i.e., moist or dry, smooth or wrinkled, raised or flat), and pigmentation. In nutrient broth, observations were made of turbidity, sediment, and pellicle formation. The nutrient broth employed contained 5 g. of peptone and 3 g. of beef extract per liter of distilled water; the nutrient agar contained in addition 20 g. of agar. Unless otherwise stated, incubations were made at room temperature, commonly about 28° C.

Growth and pigmentation were also observed on potato. Large potatoes were cut cylindrically with a 20 mm. cork borer. These cylinders were cut diagonally and placed in 2 oz. screw-cap bottles, together with about 5 ml. of distilled water. Sterilization was with steam at 15 lbs. pressure for 20 minutes, and with caps screwed tightly during sterilization. Inoculated potato slants were incubated at room temperature. Observations for growth and pigmentation were made after 4, 10, and 30 days.

Ability to grow without organic nitrogen was observed both on the sodium nitrate-dextrose agar of Dimmick (25) and on the ammonium phosphate agar of Smith *et al.* (51) when enriched with dextrose.

Ability to grow in the presence of 5.0, 7.5, and 10.0 per cent sodium chloride was determined in nutrient broth cultures incubated at room temperature.

Ability to grow at 37° and 45° C. also was observed in nutrient broth cultures. Uninoculated broth tubes were pre-incubated at the appropriate temperature. They were removed from the incubator for only a very few minutes at the time of inoculation. Incubation temperatures of 37° or 45° C. were shown by thermometers immersed in small containers of distilled water placed at the level of the inoculated broth tubes.

Ability to survive exposure to a temperature of 65° C. for 30 minutes was determined for nearly all cultures. A large water bath thermostatically-controlled was employed. Milliliter aliquots were taken from nutrient broth cultures and placed in tubes containing 9 ml. of distilled water, and which previously had reached equilibrium with the water bath. Tubes were returned to the water bath as quickly as possible, and timing of the exposure interval started. At the termination of the exposure, 1 ml. of the material was pipetted directly to a sterile petri dish and mixed with 9 ml. of melted nutrient agar, whose temperature was 45° C. A second milliliter was pipetted into 99 ml. of sterile water. After thorough mixing, 1 ml. of liquid was withdrawn and mixed with melted agar. Control plates with unheated

inocula were prepared simultaneously. Colony counts were taken after 7 days of incubation.

Media and Procedures for Physiological Studies

Gelatin hydrolysis was determined by the method of Smith et al. (51). A medium containing 4 g. of gelatin, 2 g. of beef extract, 5 g. of peptone, 20 g. of agar, and 1 l. of distilled water was employed. Incubation was at room temperature or approximately 28° C. for 7 days. Plates were then flooded with a reagent consisting of 20 ml. of concentrated HCl, 15 g. of $HgCl_2$, and 100 ml. of distilled water. Colony growth was gently rubbed from the medium, and action of the reagent allowed to continue for about 30 minutes. Clear zones were interpreted as evidence of gelatin hydrolysis.

Casein hydrolysis was determined by the method of Smith et al. (51). A medium consisting of 500 ml. of skim milk, 500 ml. of distilled water, and 10 g. of agar was employed. Skim milk was prepared by dissolving 50 g. of dehydrated skim milk in 500 ml. of cold distilled water. The agar and skim milk were autoclaved in separate containers at 15 pounds steam pressure for 20 minutes, cooled to 45° C., and mixed. Cultures were streaked on plates of the solidified agar and incubated at room temperature for 7 days. Clearing of the medium under or around the line of streak was interpreted as casein hydrolysis.

The production of urease was determined as follows: 2 ml. of distilled water were added to a culture that previously had grown on

a nutrient agar slant for 7 days at room temperature. The organisms were gently swept from the surface of the agar with a sterile wire loop, and mixed thoroughly with the added water. The suspension was divided into two equal parts, and each part transferred to a clean test tube. One drop of 0.04 per cent phenol red solution was added to each tube, and the reaction brought to pH 7 by adding either 0.01 N HCl or 0.01 N NaOH. A few milligrams of urea were added to one tube; the other was kept as a control. Development of a distinctly red color within 30 minutes in the tube containing urea, together with absence of red color in the control, was interpreted as a positive test for the presence of urease.

The ability of organisms to hydrolyze uric acid was determined according to the method of Smith et al. (51). The uric acid agar had the following composition: uric acid, 1 g.; NH_4NO_3, 1 g.; K_2HPO_4, 0.5 g.; KCl, 0.2 g.; $MgSO_4 \cdot 7H_2O$, 0.2 g.; agar, 15 g.; and distilled water, 1 l.. The uric acid, being very insoluble in water, remained dispersed in the agar medium in a crystalline form. Cultures were incubated at room temperature and observed daily for 7 days. Disappearance of the crystals under and around colonies was interpreted as uric acid hydrolysis.

The method of Ayers and Rupp (2) was used for determining sodium hippurate hydrolysis. Tubes were prepared containing 5 ml. of the following broth: beef extract, 2 g.; peptone, 5 g.; sodium hippurate, 10 g.; and distilled water, 1 l.. The level of the medium was marked on each tube immediately following steam sterilization. Following

inoculation, cultures were incubated at room temperature for 4 days, and the broth was restored to volume by adding the necessary amount of distilled water. By means of a graduated pipette, 0.8 ml. of the broth culture was transferred to a clean test tube. To this portion was added 0.2 ml. of a test reagent prepared by dissolving 12 g. of ferric chloride in 100 ml. of 2 per cent HCl. The formation of a permanent precipitate was considered as evidence of sodium hippurate hydrolysis.

The ability of cultures to reduce nitrates to nitrites was determined on Dimmick's medium (25). Its composition is as follows: K_2HPO_4, 0.5 g.; NaCl, 0.5 g.; $MgSO_4 \cdot 7H_2O$, 0.2 g.; $NaNO_3$, 2 g.; dextrose, 10 g.; agar, 15 g.; and distilled water, 1 l.. Because of poor or no growth of some bacterial cultures on this substrate, all cultures were also grown on an enriched form of the medium, which contained in addition 2 g. of peptone per liter. Cultures were incubated at room temperature for 7 days. Four drops each of Wallace and Neave's (59) reagents A and B were then added. Reagent A consisted of 8 g. of sulfanilic acid in 1 l. of dilute sulfuric acid (1 part concentrated H_2SO_4 in 20 parts of water). Reagent B consisted of 6 ml. of dimethylalphanaphthylamine in 1 l. of 30 per cent acetic acid. The appearance of a pink or red color within 30 minutes following reagent additions was taken as an indication that nitrites were present.

Production of ammonia was determined in two peptone broth media, one of which contained 40 g. of proteose-peptone and 1 g. of dextrose per liter, and the other, 10 g. of proteose-peptone and 0.25 g. of

dextrose. Inoculated cultures were incubated for 4 and 10 days at 28° C.. After incubation, 3 drops of Nessler's solution (1) were added to each tube and the intensity of the color that developed was compared with that developed by the reagent in uninoculated controls.

The production of indol was observed on a medium containing 30 g. of peptone, 3 g. of beef extract, 0.2 g. of peptonized iron, 0.025 g. of sodium thiosulfate, 3 g. of agar, and 1 l. of distilled water. After stab inoculation of the tubed medium, 1/4 x 1 inch strips of filter paper, previously soaked in saturated oxalic acid solution and dried, were suspended above the medium by securing the strips between the mouth of the tube and the inserted cotton plug. The filter paper was observed daily for 1 week for pink or red color indicating indol production. After 8 days of incubation, cultures were tested with Ehrlich's reagent (49), consisting of 1 g. of paradimethylamino-benzaldehyde, 95 ml. of 95 per cent ethyl alcohol, and 20 ml. of concentrated HCl. In performing the test, about 2 ml. of ethyl ether were added to the tube, shaken with the contents, and allowed to stand for approximately 8 minutes. Then 1.0 ml. of Ehrlich's reagent was allowed to run slowly down the inside of the tube in such a manner that it layered betweed the culture medium and the supernatent ether. The development of a purplish red color at the intersurface within 5 minutes was interpreted as a positive test for the presence of indol.

The production of hydrogen sulfide was observed on agar slants of the following composition: peptone, 15 g.; proteose-peptone, 5 g.; dextrose, 1 g.; lead acetate, 0.2 g.; sodium thiosulfate, 0.08 g.;

agar, 15 g.; and water, 1 l.. The inoculum was streaked on the surface of the slant and also stabbed into the butt of the agar. Incubation was at room temperature; observations for darkening of the medium were made at 7 and 21 days.

Catalase production was determined on cultures grown on nutrient agar slants for 4 days. Surface growth was flooded with a few milliliters of 3 per cent hydrogen peroxide. Evolution of gas bubbles was interpreted as a positive test for catalase production.

The production of acetylmethylcarbinol was determined in broth containing 7 g. proteose-peptone, 5 g. dextrose, and 5 g. NaCl per liter. The initial reaction was adjusted to 6.8. After incubation for 7 days, there was added to the broth an equal volume of 40 per cent NaOH, and also a few milligrams of creatin. The tubes were shaken and then incubated at 37° C. for 2 hours. The appearance of a red color was interpreted as a positive test for acetylmethylcarbinol. Acidity production was determined in separate tubes of the same broth by means of a glass electrode assembly. These pH reactions were checked in several duplicate series of tests in which brom thymol blue indicator was employed in place of the glass electrode.

Citrate utilization was determined on a medium containing 2 g. of sodium citrate, 0.5 g. of KH_2PO_4, 2 g. of NH_4NO_3, 15 g. of agar, 10 ml. of 0.04 per cent phenol red solution, and 1 l. of distilled water. Reaction of the medium was adjusted to pH 6.8 prior to the addition of the phenol red. Inoculated slants were incubated at room temperature for 10 days and observed daily for growth and for the appearance of

a red color indicative of citrate utilization.

Starch hydrolysis was determined on a medium containing 10 g. of starch, 2 g. of beef extract, 5 g. of peptone, 20 g. of agar, and 1 l. of water. Agar plates were streaked in duplicate and incubated at room temperature for 7 days. One plate of each pair was flooded with 95 per cent ethyl alcohol (51) and allowed to stand about 20 minutes. A clear zone remaining under and around the line of growth was interpreted as evidence of starch hydrolysis. The duplicate plate was flooded with Lugol's iodine solution (1) and observed after five minutes. In both instances, the organisms were gently rubbed from the agar immediately after the alcohol or iodine solution was added in order to insure contact between developer and medium. Cultures that gave clear zones with alcohol and failed to give colorless zones with iodine were transferred to a medium in which starch was the only source of organic carbon. This medium contained 10 g. of potato starch, 0.5 g. of K_2HPO_4, 0.5 g. of NaCl, 0.2 g. of $MgSO_4 \cdot 7H_2O$, 2 g. of $NaNO_3$, 20 g. of agar, and 1 l. of distilled water. Duplicate plates were inoculated, incubated at room temperature for 21 days, and developed with alcohol and iodine as described above.

Fermentation studies were made on a medium containing 1 g. of $NH_4H_2PO_4$, 0.2 g. of K_2HPO_4, 0.2 g. of $MgSO_4 \cdot 7H_2O$, 20 g. of agar, 10 g. of a test carbohydrate, 20 ml. of 0.04 per cent brom cresol purple solution, and 1 l. of distilled water. The initial reaction was adjusted to 6.8. Tubed media were steam sterilized at 10 pounds pressure for 10 minutes, and pre-incubated to determine sterility

before inoculation. Inoculated cultures were observed daily for 10 days for growth and for change in color of indicator. For certain cultures not using inorganic nitrogen, an enriched form of the medium containing 2 g. of tryptone per liter was employed.

EXPERIMENTAL RESULTS

Observations on Motility and Morphology

Direct microscopy

By direct microscopy with the light microscope, motility was observed for 10 of the 26 cultures of *Arthrobacter* employed for study. Cultures 7 and 9, received as *Arthrobacter simplum*, culture 13, received as *Corynebacterium simplex*, cultures 15-17 inclusive, received as *Bacterium globiforme*, and cultures 18-21 inclusive, were motile. All other cultures of group I were nonmotile. No cultures of *Jensenia* or *Nocardia* (group II), of the animal diphtheroids (group III) or of *Microbacterium* (group VI) were motile. The majority of the cultures of cellulolytic and phytopathogenic corynebacteria were motile; only cultures 47 and 51 of group IV, and culture 56 of group V, failed to show motility.

In the examination of wet mounts prepared from young nutrient broth cultures, cell branching was commonly noted for bacteria in each of the six groups. Branching was most commonly observed in cultures of the *Jensenia-Nocardia* group, and least commonly noted in *Microbacterium*. The extent of cell branching in the remaining four groups was intermediate between the two extremes just noted, and it was not possible to differentiate bacteria of groups I, III, IV and V on the characteristic of cell branching.

Examination of cells *in situ* on thin agar films revealed the occurrence of branched cells for bacteria representative of each of

the six groups, and in addition yielded information on cell shape and arrangement in extremely young colonies. Observations were made on the following cultures: 3, 9, 15, 27, 31, 33, 34, 38, 42, 43, 48, 49, 52, 56, 57, 58, and 60.

Nocardia rubra and Jensenia canicruria (cultures 31 and 27, respectively) showed very distinctly the formation of an early mycelium. Nocardia rubra developed an early mycelium consisting of a single cell branched repeatedly and measuring about 50 μ in diameter. Fragmentation within the mycelium then occurred, but only very slowly, and there was little change in the colony appearance until its diameter had reached roughly 200 μ.. In colonies of this size, fragmentation became sufficiently frequent to enable the resulting short fragments or "cells" to become contiguous and to commence piling up in the central or oldest portion of the colony. A smooth or bacterial-type colony appearance was thus initiated in the center of the mycelium. Long, profusely branched cells continued to radiate from the margins of these colonies for at least an additional 24 hours. Photomicrographs of colonies of Nocardia rubra 31 are shown in Fig. 1.

Jensenia canicruria developed in similar fashion, with the exception that fragmentation occurred somewhat more rapidly. It could be observed in single-cell mycelia by the time that they had attained diameters of 20 μ, and it was commonly observed in colonies 100 μ. in diameter. Photomicrographs of Jensenia canicruria 27 are shown in Fig. 2.

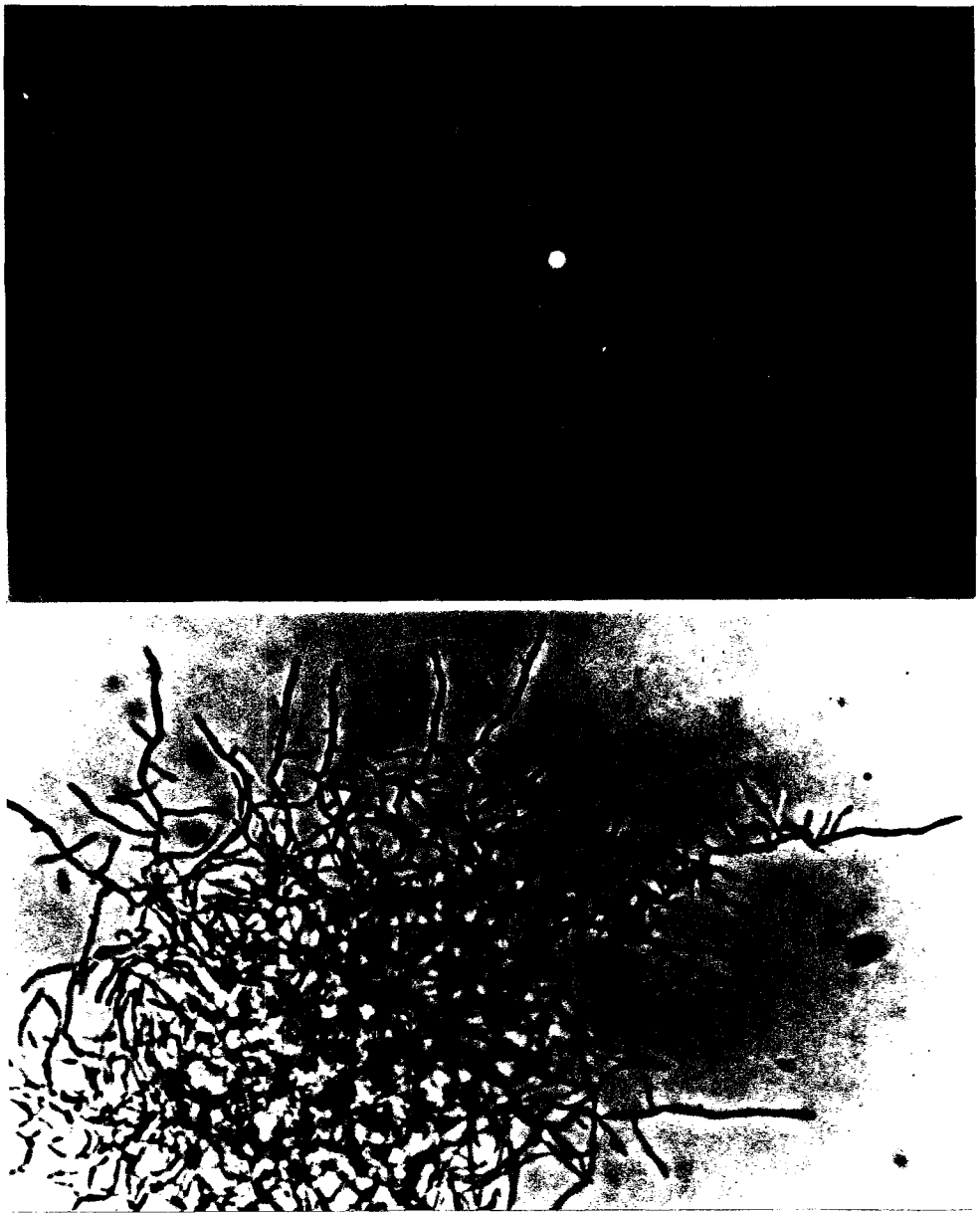

Fig. 1. Micro-colonies of Nocardia rubra 31.

Upper: 12 hours after seeding. X 1200.
Lower: 30 hours after seeding. X 600.

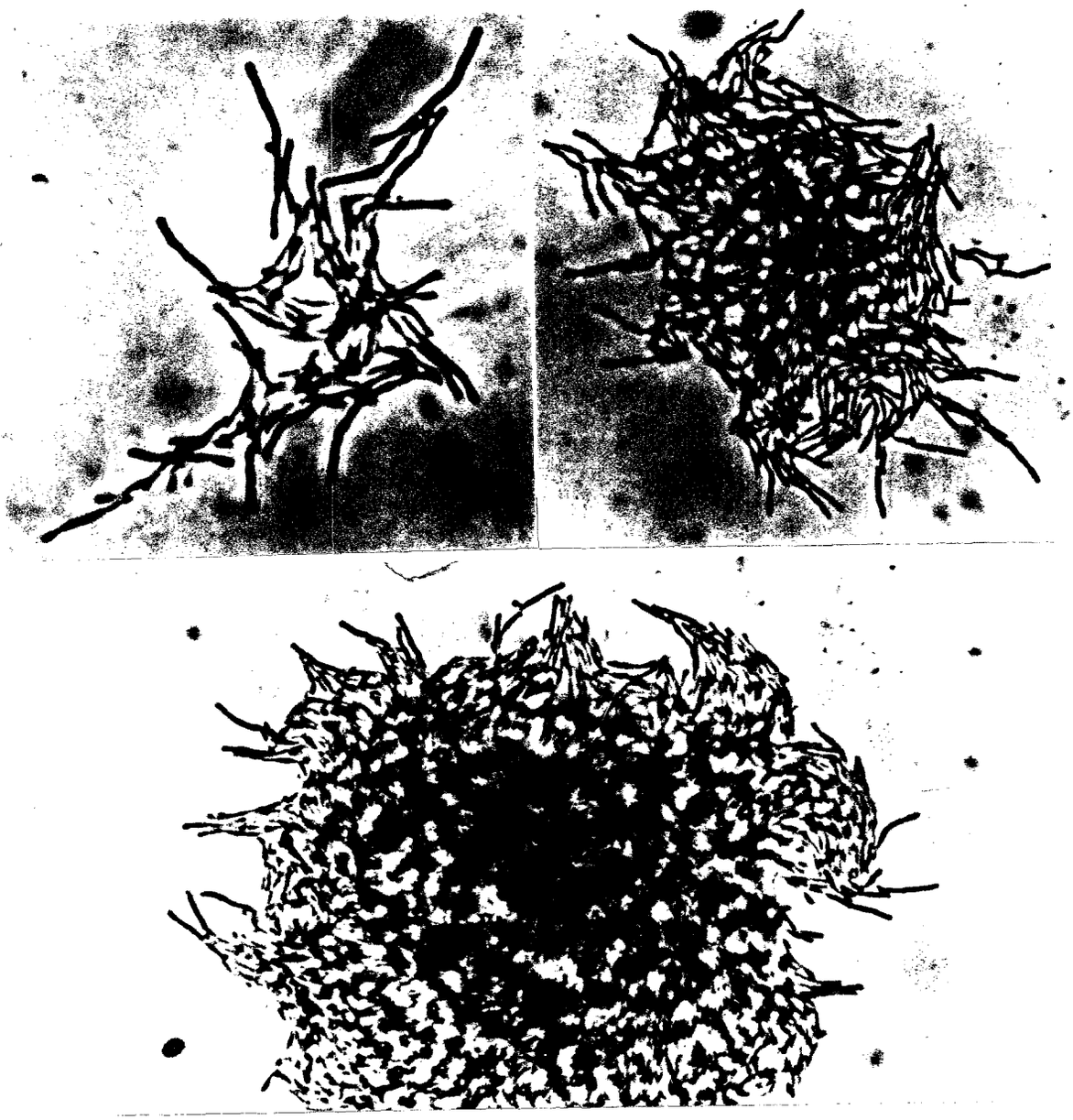

Fig. 2. Micro-colonies of Jensenia canicruria 27.

Upper left: 20 hours after seeding. X 1200.
Upper right: 24 hours after seeding. X 600.
Bottom: 30 hours after seeding. X 600.

The Arthrobacter, animal diphtheroid, cellulolytic and phyto-pathogenic corynebacteria, and the microbacteria cultures examined failed to reveal any noteworthy differences among themselves in cell size or shape, or in appearance of extremely young colonies. All 5 groups distinguished themselves from Nocardia and Jensenia in that an open mycelium was not formed initially. Fragmentation occurred in cells very shortly after their initial budding and branching, and individual cells seldom exceeded 10 μ in length when viewed in situ on agar surfaces. Only in cultures 10, 33, and 42 were cells up to 15 μ in length encountered.

In culture groups I, III, IV, V, and VI, cell division was sufficiently early and sufficiently rapid to prevent formation of an early, open mycelium. Cells remained adjacent to one another from the time of onset of colony formation, and formed round, smooth micro-colonies whose initial diameters were of the order of 10 to 15 μ. The single exception was culture 33, which was observed to form a partially fragmented mycelium exceeding 20 μ in diameter. This appearance was very transitory, and colonies 30 μ in diameter appeared as smooth colonies.

Photographs of micro-colonies of Arthrobacter and Corynebacterium are shown in Figs. 3 and 4.

Stained cell smears

Examination with the light microscope of cells taken from nutrient broth and stained with the tannic acid-crystal violet technique failed

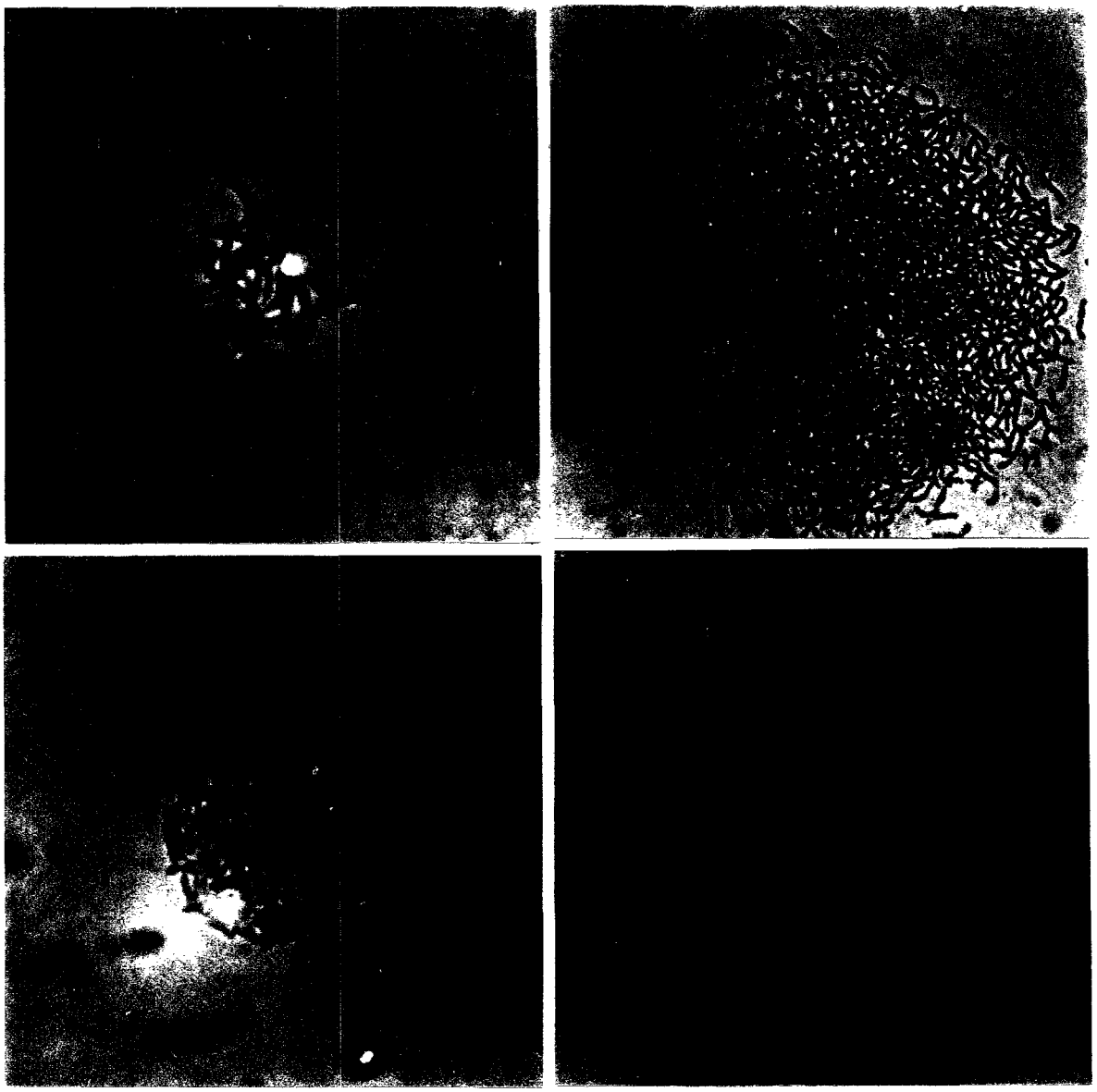

Fig. 3. Micro-colonies of Arthrobacter.

Upper left: A. globiforme 4. 12 hours after seeding. X 1200.
Upper right: Same. 24 hours after seeding. X 600.
Lower left: A. helvolum 10. 12 hours after seeding. X 1200.
Lower right: Same. 24 hours after seeding. X 600.

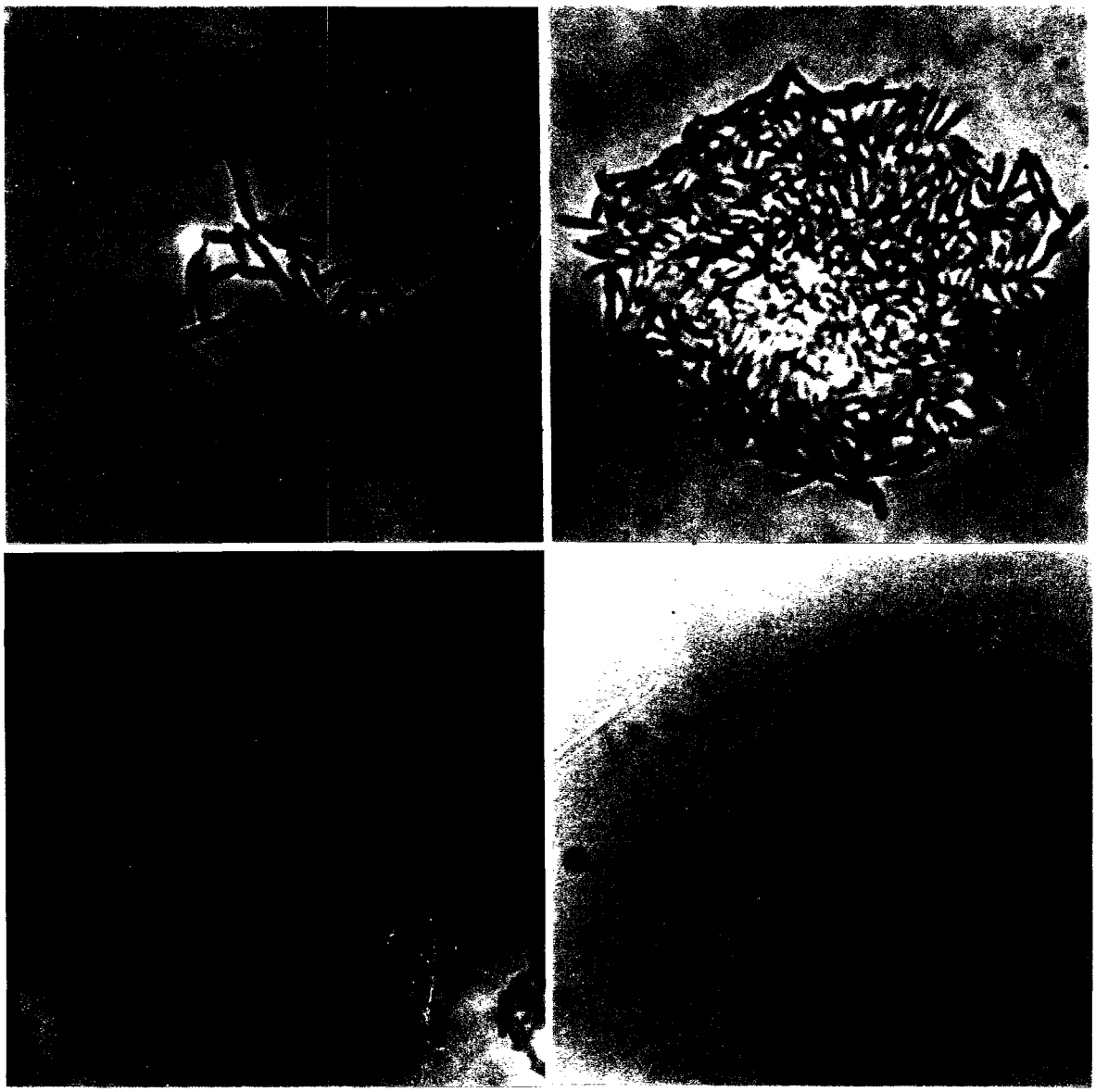

Fig. 4. Micro-colonies of Corynebacterium.

Upper left: C. equi 33. 12 hours after seeding. X 1200.
Upper right: Same. 20 hours after seeding. X 1200.
Lower left: C. flaccumfaciens 55. 18 hours after seeding. X 1200.
Lower right: Same. 24 hours after seeding. X 600.

to reveal any cultures that failed to contain both multi-celled and single-celled units. The observation of Bisset and Moore (9, 10) that "single" cells of corynebacteria of animal origin were in fact compound while single cells of Jensenia canicruria and of the soil diphtheroids generally were truly single units was not confirmed. The differences actually encountered appeared simply to reflect the tendency of cultures to form and to retain early mycelia. Non-fragmenting long cells were most commonly seen in Jensenia and Nocardia.

The cell wall staining technique revealed that corynebacteria from soil, plant, or animal sources were extremely pleomorphic in young subcultures, and that single cells could appear either as cocci, rods, or branched rods. "Compound" cells appeared to be associated with the phenomenon of rapid cell division of the longer rods into coccoid cells. In those groups (I, III-VI) which failed to show development of an open mycelium in direct microscopic studies, "compound" cells containing 2, 3, or 4 transverse walls were encountered. But at the same time, there appeared a large number of coccoid cells. In young subcultures of Jensenia canicruria, many of the longer and larger cells of the type associated with the formation of an early mycelium failed to show internal subdivisions, or transverse cells walls. At this stage of growth, very few coccoid cells were present. At the time of onset of smooth colony formation, short coccoid cells became predominant, and compound cells also could be seen present.

Gram staining responses of the 26 cultures in the Arthrobacter group were variable. This variation appeared to depend partly on the

length and conditions of incubation and partly on unexplained differences between individual strains. Eleven of the 26 cultures appeared as Gram-negative slender rods after 14 hours of growth on nutrient agar, and as Gram-positive, short coccoid rods after 5 days. Some strains appeared predominantly Gram-negative in both young and old subcultures. Strains which most nearly fitted the Gram staining pattern described by Conn (18), that is, Gram-negative when young but Gram-positive on aging, were cultures: 1, 4, 8, 9, 11, 14, 22, 23, 24, 25, and 26. Strains commonly seen as Gram-negative in both young and old subcultures were 5 and 7. In the Jensenia-Nocardia group of 5 cultures, strain variation in Gram response was also encountered. Cultures 27, 28, and 31 were Gram-positive in both young and old subcultures, and cultures 29 and 30 were Gram-negative at 14 hours and after incubation periods as long as 3 days.

The Gram responses of the cellulolytic and phytopathogenic corynebacteria and of the animal diphtheroids did not distinguish them as subgroups from each other or from the Arthrobacter group of cultures. The statement of Conn and Dimmick (21) that the animal diphtheroids usually are Gram-positive when young and Gram-negative on aging was not confirmed. Their statement that Corynebacterium poinsettiae, C. michiganense, and C. flaccumfaciens are commonly Gram-positive was confirmed. At 14 hours, these species showed Gram-positive but somewhat unevenly staining pleomorphic rods; at 5 days, their cells appeared as Gram-positive elongated cocci and short rods.

Cultures 49, 50, and 51 of the cellulolytic group were Gram-negative at both 14 hours and 5 days; 53 was Gram-negative at 14 hours but Gram-positive at 5 days; and 46, 47, 48, and 52 were Gram variable at both ages. All the cellulolytic corynebacteria were very pleomorphic. Their cells were frequently curved and irregular in outline. Clubbed and branching cells were commonly present in young subcultures. Older subcultures consisted of elongated cocci, and short and medium rods. There were usually fewer coccoid cells than rods, in contrast to many cultures of the Arthrobacter group which in older subcultures appeared almost entirely as cocci.

Electron microscopy

Electronographic study of representative species of Arthrobacter, Corynebacterium, and Cellulomonas, and of Jensenia canicruria and of Microbacterium lacticum, revealed that all these species contained round or nearly round electron-opaque inclusion bodies. These opaque bodies were visible within cells viewed in a 50 kv electron beam. Pre-treatment of cells with acid or alkali solutions, or growth on other than ordinary culture media, were not prerequisite steps. Although single cells frequently contained but single opaque inclusions, some contained plural inclusions, and as many as 16 opaque bodies were counted in a "cell filament" of Arthrobacter globiforme.

The cell inclusions observed in corynebacteria of soil, plant, and animal origin, and in cellulolytic corynebacteria, were indistinguishable in size, frequency, and general appearance. Microbacterium

lacticum resembled the soil corynebacteria both in its cell shape and outline and in its cell inclusions.

Electron micrographs showing the occurrence of cell inclusions in bacterial cultures representative of groups I, II, IV, V, and VI are presented in Figs. 5-10, inclusive.

Certain bacterial strains, in the past commonly known as nonmotile but currently found motile by direct microscopic examinations, were observed in cell preparations shadowed with gold prior to electron microscopy. Electron micrographs showing flagella or flagella fragments associated with cells of Arthrobacter simplum, Corynebacterium simplex, and Cellulomonas fima are shown in Figs. 11 and 12.

Examination with an electron microscope of cells harvested from nutrient broth subcultures incubated 12 to 24 hours and shadow-cast with gold prior to microscopy showed clearly the lateral branching and the elongated cells that had been observed previously in studies with a light microscope. Electron micrographs illustrative of the type of cell branching most commonly encountered among soil and other corynebacteria are presented in Figs. 13 and 14.

In agreement with the observations made on cell smears stained by the tannic acid-crystal violet method, examination with an electron microscope showed that individual cells of Jensenia conicruria at times appeared free of cross walls and at times appeared as compound cells. Single elongated cells with and without cross walls, and also short rods or coccoids of the type which appeared following fragmentation of the early mycelium or of elongated young cells are shown

Fig. 5. Electron Micrograph of Arthrobacter simplum 7. Cells from nutrient agar 2 days after seeding. x 21,000.

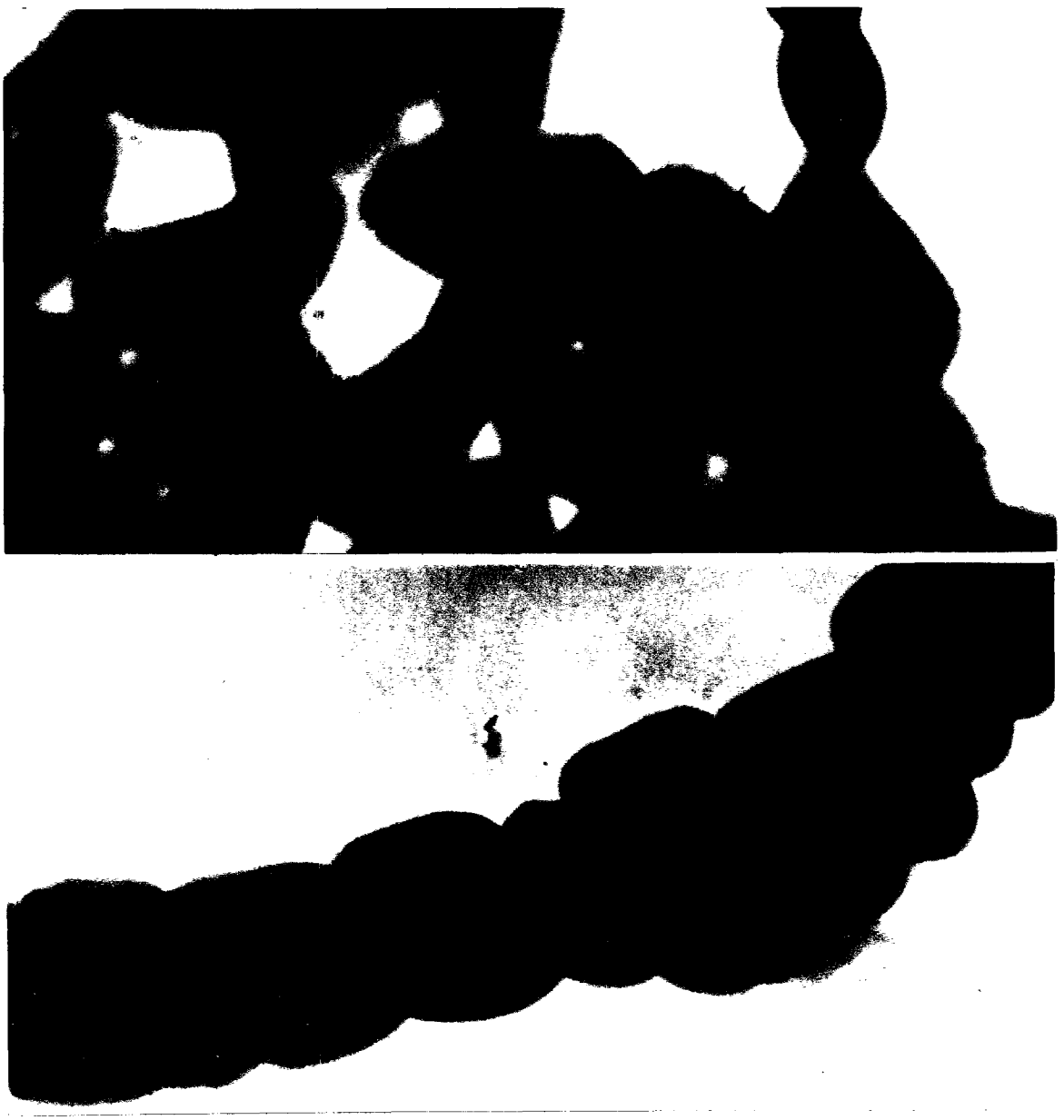

Fig. 6. Electron Micrographs of Arthrobacter.

Upper: A. tumescens 12. From nutrient agar 2 days after seeding. X 31,500.
Lower: A. simplex 13. From nutrient agar 3 days after seeding. X 31,500.

Fig. 7. Electron Micrograph of Cellulomonas flavigena 51. Cells from nutrient agar 3 days after seeding. X 21,000.

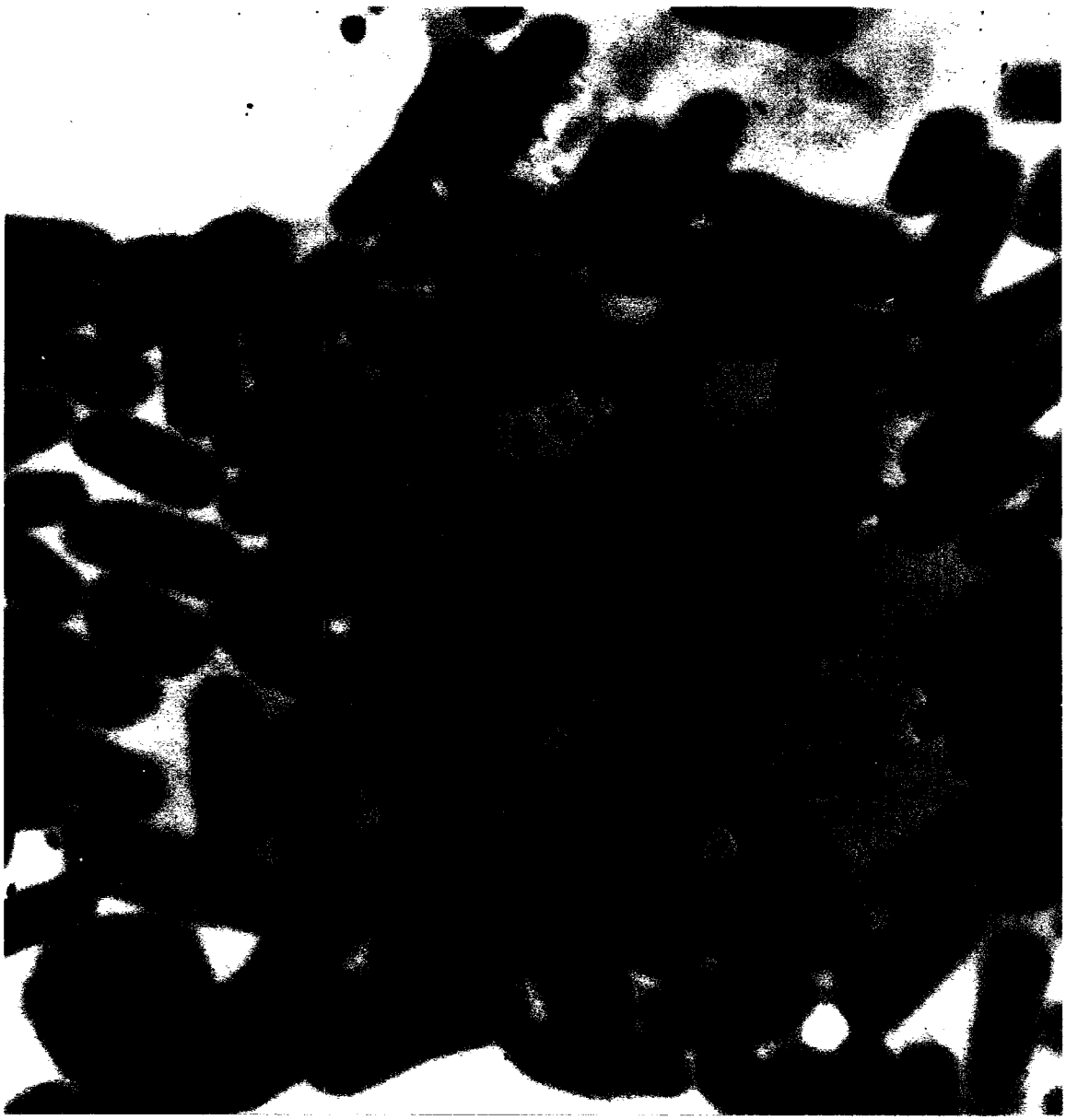

Fig. 8. Electron Micrograph of Microbacterium lacticum 58.
Cells from nutrient agar 4 days after seeding.
X 21,000.

Fig. 9. Electron Micrograph of Corynebacterium flaccumfaciens 55. Cells from nutrient agar 4 days after seeding. X 21,000.

Fig. 10. Electron Micrographs of *Jensenia* and *Corynebacterium* Cultures.

Upper: *J. canicruria* 27. From nutrient agar after 3 days. X 21,000.
Lower: *C equi* 33. From nutrient agar after 3 days. X 21,000.

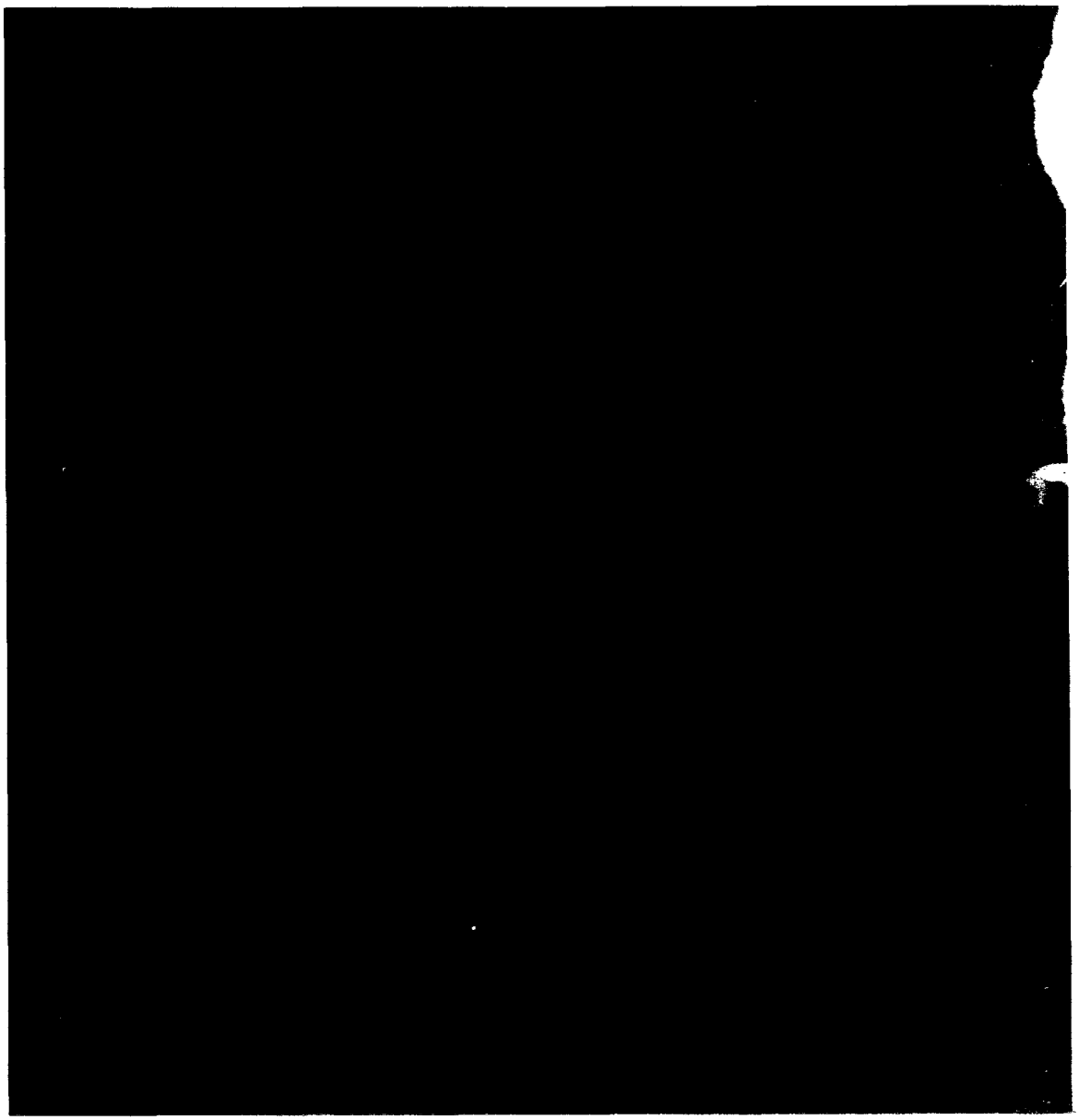

Fig. 11. Electron Micrograph of Flagellated Cell of Arthrobacter simplum 7.

Cell taken from nutrient broth after 24 hours; shadow-cast with gold. X 21,000.

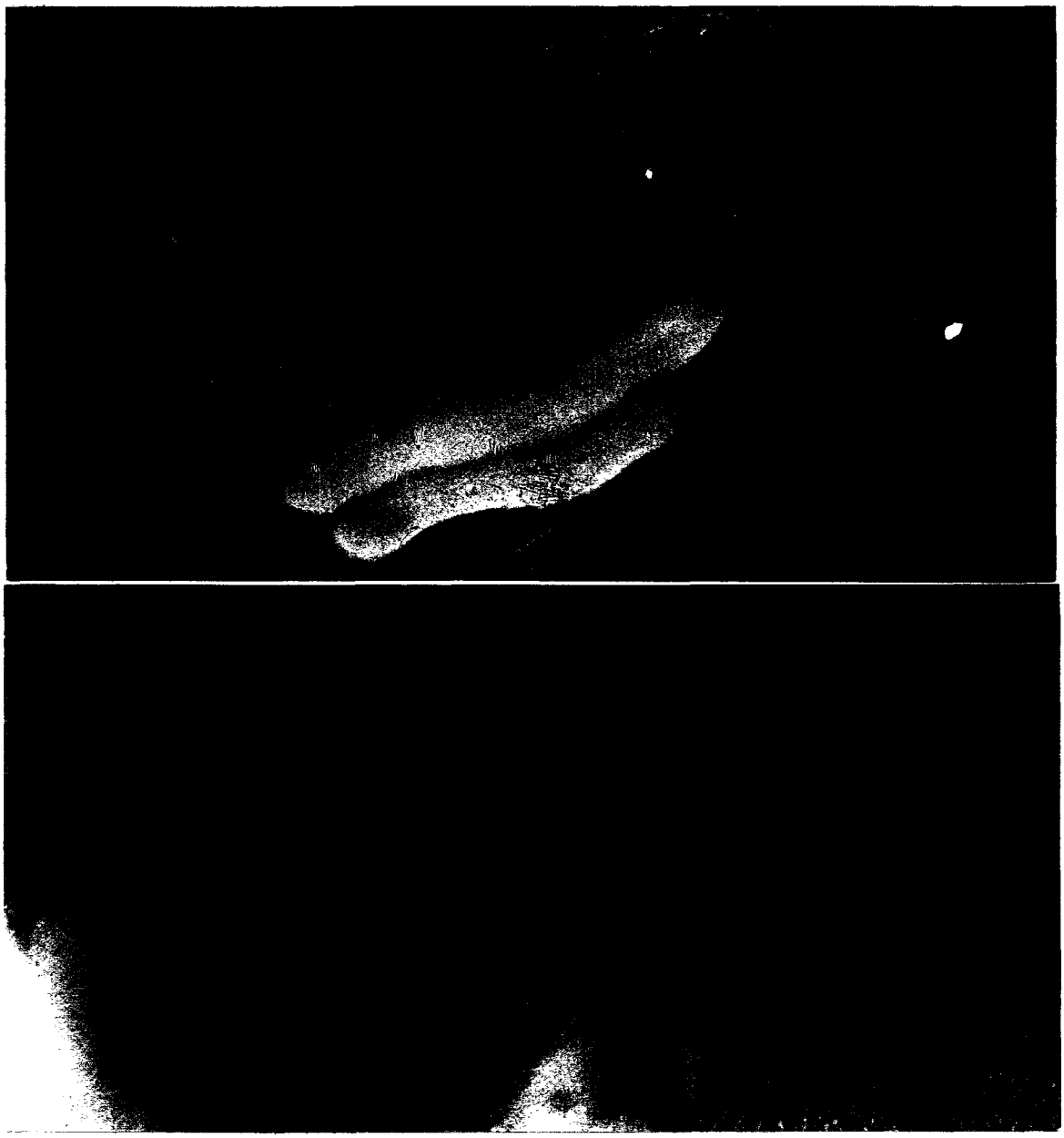

Fig. 12. Electron Micrographs of Flagellated Cells of Cellulomonas and Corynebacterium Cultures.

Upper: Cellumonas fimi 46. From nutrient broth after 18 hours; shadow-cast with gold. X 31,500.

Lower: Corynebacterium simplex 13. From nutrient broth after 26 hours; shadow-cast with gold. X 31,500.

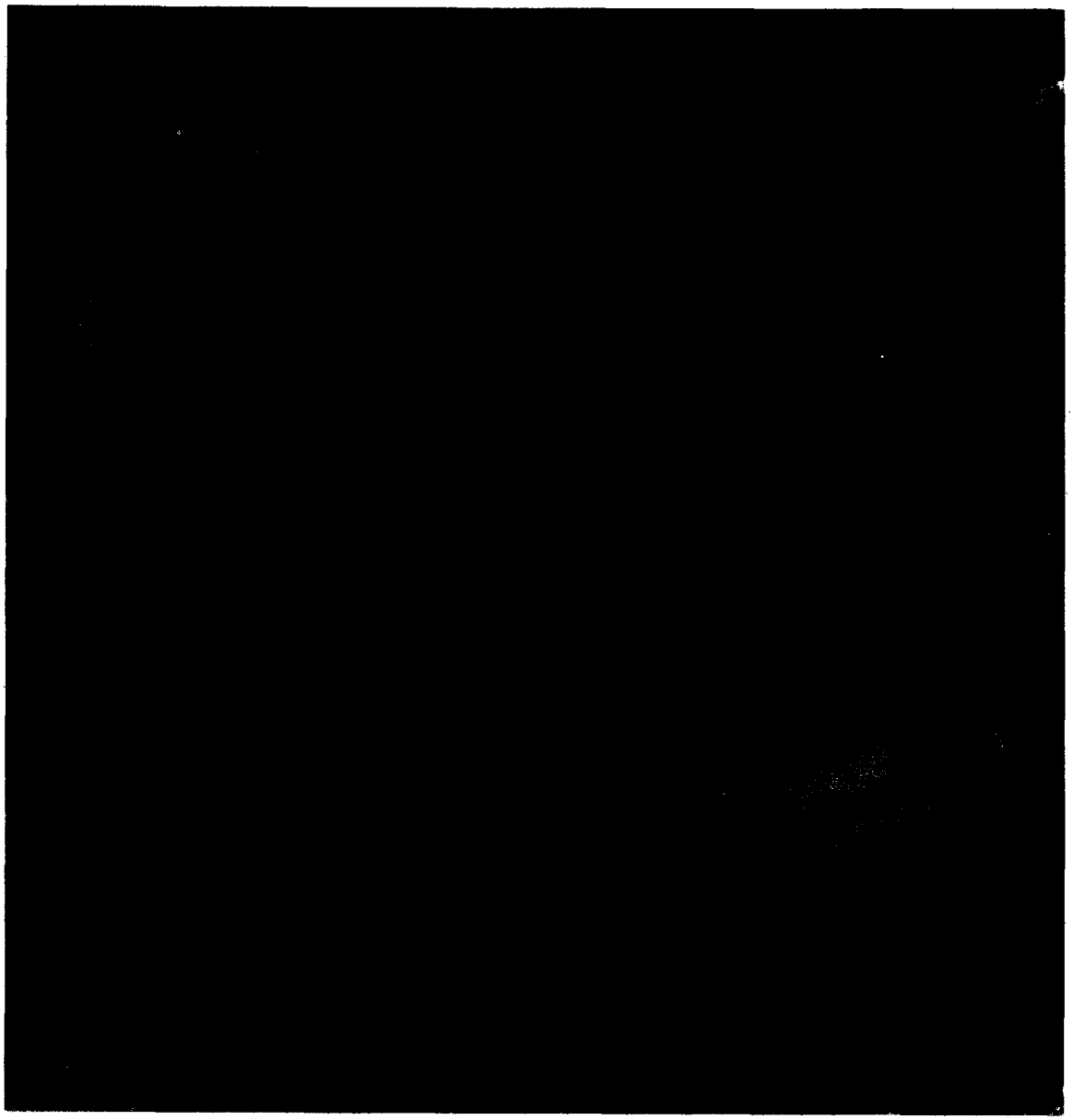

Fig. 13. Electron Micrograph of Branching Cell of Arthrobacter simplum 9. Cells taken from nutrient broth after 22 hours; shadow-cast with gold. X 21,000.

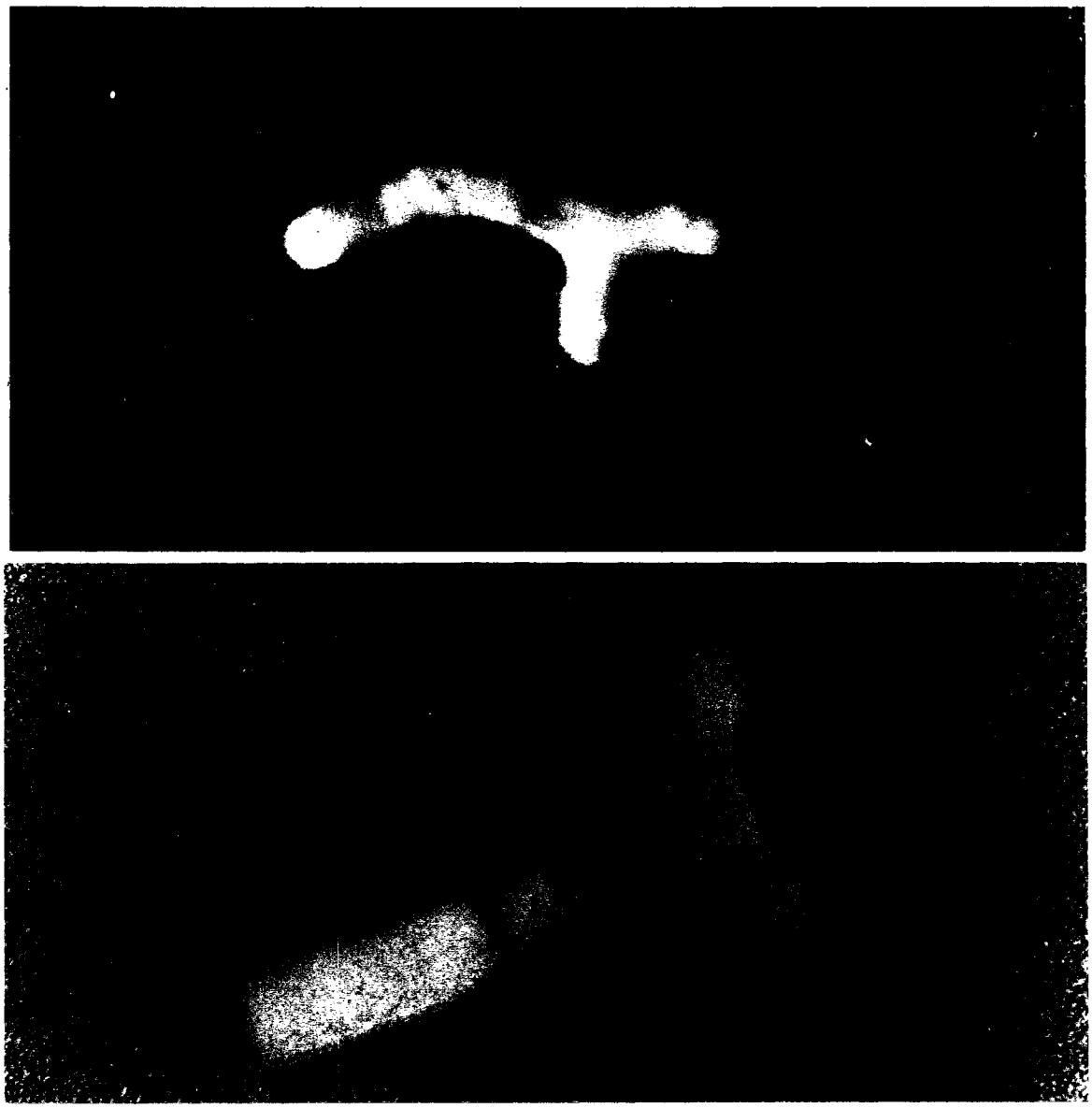

Fig. 14. Electron Micrographs of Branching Cells of Corynebacterium and Cellulomonas Cultures.

Upper: Corynebacterium michiganense 56. From nutrient broth after 24 hours; shadow-cast with gold. X 21,000.

Lower: Cellulomonas fimi 46. From nutrient broth after 18 hours; shadow-cast with gold. X 31,500.

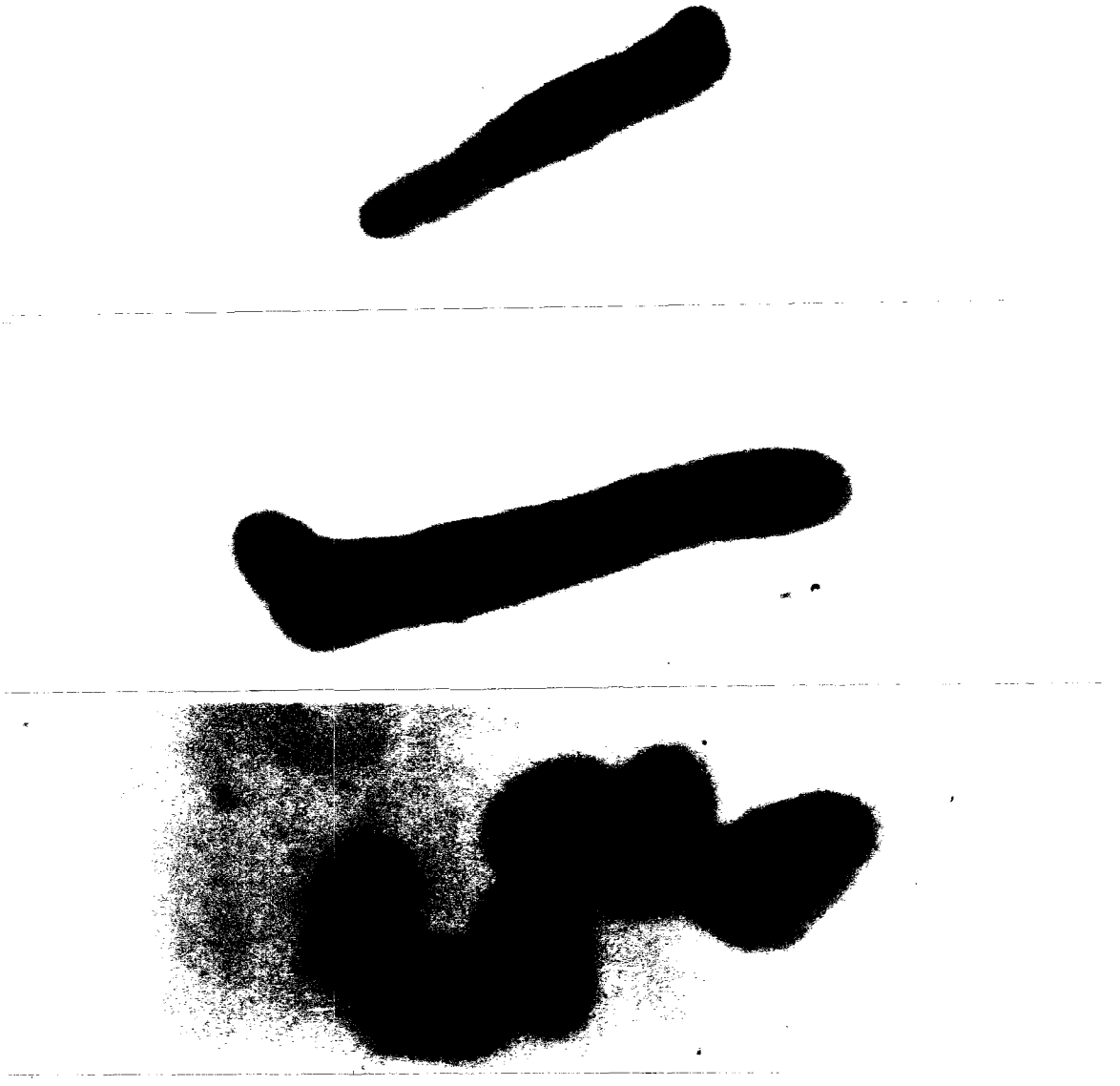

Fig. 15. Electron Micrographs of Jensenia canicruria 27.

Top: Cell from nutrient agar after 24 hours. X 21,000.
Middle: Cell from nutrient agar after 24 hours. X 21,000.
Bottom: Cells from nutrient agar after 72 hours.

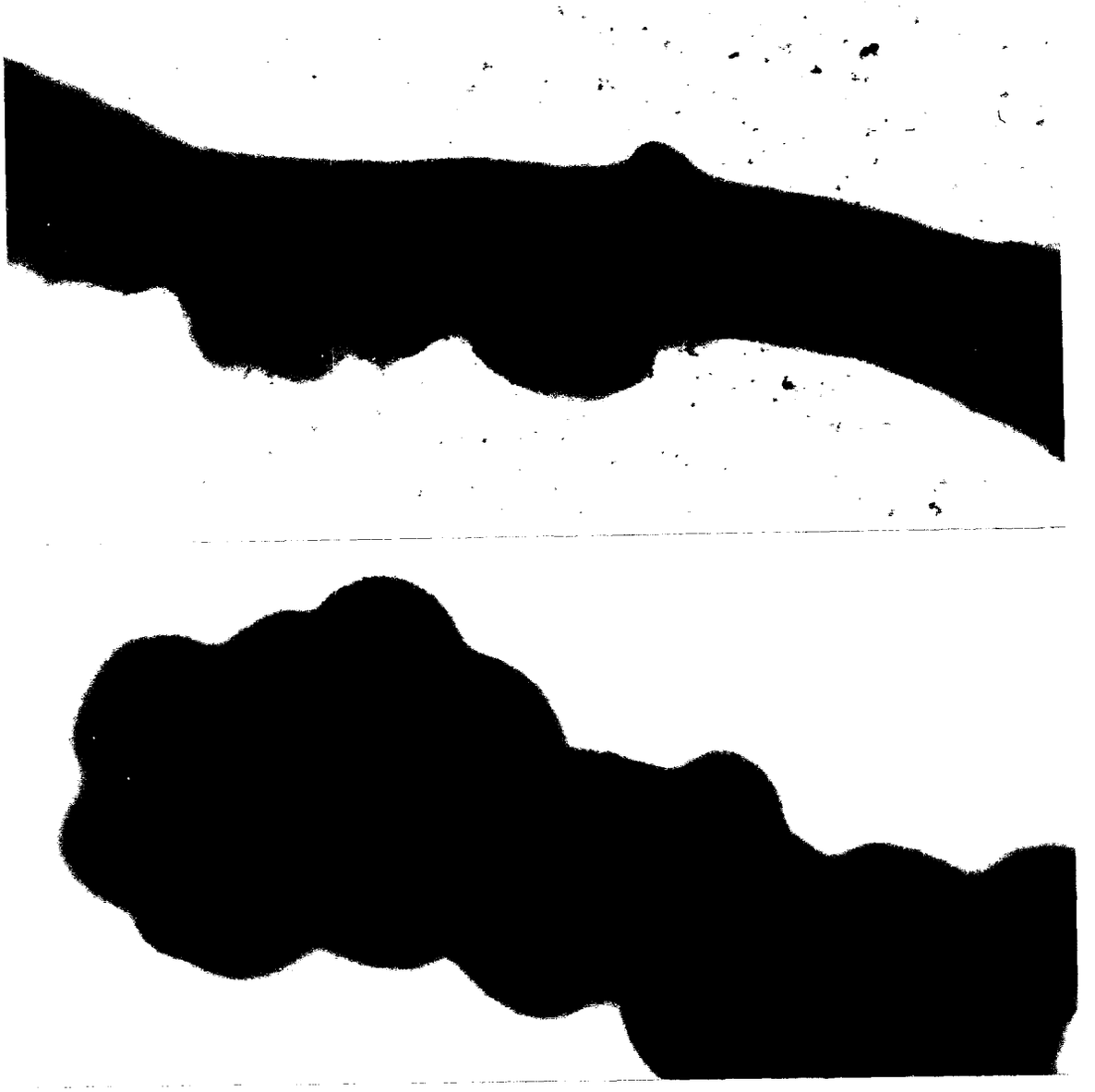

Fig. 16. Electron Micrographs of Arthrobacter globiforme 4.

Upper: From nutrient agar after 8 hours. 31,500 X.
Lower: From nutrient agar after 48 hours. 31,500 X.

in Fig. 15. An elongated and laterally budded cell of Arthrobacter globiforme, and the coccoid cells characteristically found in subcultures after 24 or more hours of growth, are shown in Fig. 16.

General Growth Characteristics

Growth on nutrient agar and in nutrient broth

All cultures of the Arthrobacter, Cellulomonas, phytopathogenic Corynebacterium, and Microbacterium groups produced raised, smooth, moist growth on nutrient agar. There were some differences among cultures in pigmentation. The pigmentation observed for individual bacterial cultures of these groups and also for groups II and III is recorded in Table 2.

Several cultures of Jensenia-Nocardia and of the animal diphtheroids produced growth that was either wrinkled or dull and dry in appearance. The agar surface growth of Nocardia rubra 31 showed sharply folded wrinkles in old subcultures. Both Jensenia canicruria 27 and the unnamed culture 28 also showed wrinkled surface growth in old subcultures. Growth in young subcultures of these three organisms was usually moist, smooth, and abundant.

Surface growth on agar of several of the animal diphtheroids was flat and of a dull and rough-looking appearance. This type of growth was noted especially for cultures 35-38, and 45.

The majority of the 60 cultures produced uniformly turbid growth in nutrient broth. Only Nocardia rubra 31 produced an entirely clear

Table 2. Pigment Production, and Extent of Growth: (a) with inorganic sources of nitrogen, (b) in the presence of sodium chloride, and (c) at 37° C.

Culture No.	Pigment produced on nutrient agar	Ability to use: Nitrate nitrogen	Ability to use: Ammonia nitrogen	Growth in presence of NaCl, per cent: 5.0	7.5	10.0	Growth at 37° C.
Group I.	Arthrobacter:						
1.	yellow	+++[a]	++	++	+g[b]	-	+
2.	yellow	+++	++	++	+g	-	+
3.	yellow	++	++	+++	+g	-	+++
4.	white	+++	+++	++	+g	-	++
5.	ivory	+++	+++	++	-	-	+++
6.	ivory	+++	+++	++	-	-	-
7.	ivory	+++	++	++g	+g	-	-
8.	ivory	+++	+++	++	-	-	-
9.	ivory	+	+	-	-	-	-
10.	yellow	++	+++	++	+g	-	+
11.	white	-	-	-	-	-	++
12.	white	-	-	-	-	-	++
13.	white	++	+++	++g	-	-	+++
14.	ivory	++	+++	-	-	-	-
15.	ivory	+	+	+	-	-	-
16.	ivory	++	++	++	-	-	-
17.	ivory	-	-	+g	-	-	-
18.	ivory	+		+g	-	-	-
19.	ivory	+	++	++g	+g	-	-
20.	ivory	++	++	++	-	-	-
21.	ivory	++	++	+g	+g	-	-
22.	ivory	+++	+++	n.d.[c]	n.d.	n.d.	-
23.	ivory	+++	+++	n.d.	n.d.	n.d.	-
24.	ivory	++	++	n.d.	n.d.	n.d.	-
25.	ivory	+++	+++	n.d.	n.d.	n.d.	-
26.	white	+++	+++	n.d.	n.d.	n.d.	-

[a]Growth responses shown as: +++ excellent; ++ moderate; + poor; - none.
[b]Symbol g indicates the growth present has a granular appearance.
[c]Not determined.

Table 2 (continued)

Culture No.	Pigment produced on nutrient agar	Ability to use: Nitrate nitrogen	Ammonia nitrogen	Growth in presence of NaCl, per cent: 5.0	7.5	10.0	Growth at 37° C.
Group II.	Jensenia-Nocardia:						
27.	ivory to slightly pink	++	++	++	+g	-	+
28.	cream	+++	+++	++	+g	-	-
29.	ivory	-	-	++	+g	-	-
30.	yellow	-	-	+	-	-	-
31.	orange red	++	++	-	-	-	+++
Group III.	Animal diphtheroids:						
32.	orange pink	+	++	+++	++g	-	+++
33.	light pink	+	++	+++	++	-	+++
34.	ivory	+	+	+++	++	+g	+++
35.	cream	-	-	+++	-	-	++
36.	ivory	-	+	+++	+++	-	+++
37.	ivory	-	+	+++	+++	++	++
38.	ivory	+	+	+++	++g	+g	+++
39.	ivory	+	+	++	++	+g	++
40.	yellow	-	-	+++	+++	+g	++
41.	yellow	-	-	+++	+++	+g	++
42.	ivory	+++	+++	+++	++	+g	+++
43.	cream	+	+	+++	+++	++	+++
44.	cream yellow	+	+	++	++	+	+++
45.	white	-	-	++	-	-	++
Group IV.	Cellulolytic corynebacteria:						
46.	yellow	-	-	-	-	-	+++
47.	ivory	+	+	+g	-	-	+++
48.	cream yellow	-	-	+g	+g	-	+++
49.	yellow	+	+	+g	+g	-	+++
50.	ivory	+	+	-	-	-	++
51.	yellow	+	+	+g	+g	-	+++
52.	white	-	-	+g	+g	-	+++
53.	ivory	+	+	+g	-	-	+++
54.	cream yellow	+++	+	+g	+g	+g	-

Table 2 (continued)

Culture No.	Pigment produced on nutrient agar	Ability to use: Nitrate nitrogen	Ability to use: Ammonia nitrogen	Growth in presence of NaCl, per cent: 5.0	7.5	10.0	Growth at 37° C.
Group V.	Phytopathogenic corynebacteria:						
55.	gold yellow	-	-	++	+g	-	+
56.	white	+	+	+g	-	-	-
57.	slightly pink	+	+	+g	-	-	++
Group VI.	Microbacteria:						
58.	light yellow	-	-	+	-	-	++
59.	white	-	-	+g	-	-	++
60.	ivory	-	-	++	+g	-	+++

broth with early pellicle formation. Cultures 27 and 28 produced either fragile pellicles or surface rings. Surface rings of growth were also noted for the following cultures: 1, 2, 5, 8, 15, 16, 17, 33, 36, 38, 39, and 44.

Growth with inorganic sources of nitrogen

Ability to show abundant growth on media containing nitrogen only in inorganic form was limited very largely to bacteria of groups I and II. Nineteen of the 26 cultures in the Arthrobacter group grew rapidly on inorganic nitrogen. Three of the five cultures of the Nocardia-Jensenia group also grew readily on such media. For the most part, corynebacteria of animal, plant, or dairy origin grew poorly or not at all on media containing only inorganic nitrogen.

The ability of the 60 bacterial cultures under investigation to utilize inorganic sources of nitrogen is shown in Table 2.

Growth on potato

Differences in the ability of cultures to grow on potato plugs were noted. Such differences appeared to depend partly on unknown variations among different lots of market potatoes. With one lot of potatoes, certain cultures could show growth or pigmentation, while on a second lot, growth or pigmentation could be entirely lacking. This variation in growth on different lots of potatoes was noted only among cultures which at most never gave more than scant growth on potato. Some bacterial cultures grew abundantly on all lots of potatoes employed.

The majority of the Arthrobacter group of cultures showed profuse growth on potato. For Arthrobacter aurescens 1, 2, and 3, and for Arthrobacter helvolum 10, growth was heavy and yellow to intensely yellow in color. Cultures 4-8, 13-14, 19, and 21-26 produced abundant growth that was creamy white to dull or greyish yellow in appearance. Cultures 9, 11, 12, 15-17, and 20 showed no growth in some tests; in others, scant growth became apparent after 4 to 10 days of incubation.

Nocardia rubra 31 produced orange red, slightly dry-appearing growth on potato; Jensenia canicruria and culture 28 produced flesh or buff colored growth which became heavily papillated upon prolonged incubation. Cultures 29 and 30 failed to grow on potato.

Of the 14 cultures of animal diphtheroids, 10 cultures showed either no growth or at best very questionable growth on potato; cultures 32 and 33 showed moderately heavy, salmon-colored growth; and cultures 42 and 43 showed moderately heavy, dirty or creamy yellow growth on potato.

The cellulolytic cultures 46-54 produced an abundant yellow growth on potato, with some darkening of the water in which the potato plug was standing. C. michiganense 56 grew readily on potato; its growth was creamy yellow in appearance. C. poinsettiae 57 grew readily on potato; its growth was salmon to flesh-colored in appearance. Microbacterium lacticum 58 and 59 and M. lacticum var. liquefaciens 60 produced scant to moderate growth on potato without pigment formation.

Growth in the presence of 5, 7.5, and 10 per cent sodium chloride

Members of the six groups of bacteria appeared about equally tolerant of the presence of sodium chloride in nutrient broth media. All 60 of the bacterial cultures grew in the presence of 5 per cent NaCl. This amount of salt was not entirely without effect on macroscopic appearance of growth. Several bacteria cultures showed less uniform turbidity in the presence of 5 per cent NaCl than they did in control broth. The granular appearance of growth, together with an increase in amount of sediment, was shown quite generally by bacteria growing in the presence of 7.5 per cent NaCl. The flocculating effect of salt was apparent in all six groups of bacteria. The majority of the bacterial cultures failed to grow in 10 per cent NaCl. As a group, the animal diphtheroids appeared most tolerant of this concentration of salt.

The growth responses of the individual bacterial cultures in broths containing 5, 7.5, and 10 per cent NaCl are shown in Table 2.

Growth at 37° and 45° C.

Roughly one-fourth of the total number of bacterial cultures failed to grow at 37° C. Almost without exception the bacteria failing to grow were members of the Arthrobacter or of the Jensenia-Nocardia groups. Eleven of 26 cultures of Arthrobacter and 3 or 5 cultures of Jensenia-Nocardia showed no growth at 37° C. Among the cellulolytic and phytopathogenic corynebacteria, only 2 cultures showed no growth. All of the cultures of animal diphtheroids and of Microbacterium grew at 37° C.

The growth responses of the individual cultures when incubated at 37° C. are shown in Table 2,

With but one exception, none of the bacteria were able to grow at 45° C. Nocardia rubra 31 showed good growth accompanied by pellicle formation in broth subcultures incubated at 45° C.

Thermal resistance

Bacteria in the Arthrobacter group of cultures were unable to survive an exposure period of 30 minutes at 65° C. Thermal resistance studies were made on cultures 1-21, inclusive. Broth subcultures from which aliquots were taken for pasteurization contained from 100,000,000 to 300,000,000 microorganisms per milliliter. Following heat treatment, counts per ml. were zero in most instances, and in no instance did the colony count exceed 10 per ml.

The cellulolytic and phytopathogenic corynebacteria in groups IV and V also failed to withstand exposures to 65° C. for 30 minutes. For subcultures whose initial counts were of the order of 100,000,000 per ml., the maximum count observed following heat treatment was 60 per ml.; the majority of the counts were less than 10 per ml. All bacteria of groups IV and V were tested for thermal resistance.

In the Jensenia-Nocardia group, cultures 27-31 inclusive failed to survive heat treatment to any appreciably greater extent than did Arthrobacter cultures 1-21 inclusive. Of the animal diphtheroids, culture 40 showed 200,000 of an initial 60,000,000, and culture 42 showed 120,000 of an initial 100,000,000, cells per ml. to remain

viable following exposure to 65° C. for 30 minutes. The remaining 12 cultures of group III failed to survive exposure to 65° C. to any appreciable extent.

In contrast to groups I-V inclusive, all Microbacterium cultures of group VI showed marked thermal resistance. The number of viable cells in cultures 58 and 60 was of the same order of magnitude following heat treatment at 65° C. as the number preceding heat treatment. In culture 59, there was reduction in number, but over one million cells per milliliter, or roughly two per cent of the number initially present, survived exposure to 65° C. for 30 minutes.

Physiological Responses

Gelatin hydrolysis

All cultures of Arthrobacter named by Conn, as well as all other cultures in group I isolated elsewhere or locally, showed hydrolysis of gelatin. Similarly, all the phytopathogenic and cellulolytic bacteria and all three cultures of Microbacterium employed for study hydrolyzed gelatin. Within group II, Jensenia canicruria 27, culture 28, and Nocardia rubra 31 failed to show any hydrolysis of gelatin in 7 days. Cultures 29 and 30 gave positive responses when tested for gelatinolysis. There was also irregularity in the pattern of responses among the animal diphtheroids of group III. The majority, cultures 32, 33, 34, 36, 37, 38, 39, 40, 41, 43, 44, and 45, failed to hydrolyze gelatin, but cultures 35 and 42 were gelatinolytic.

Casein hydrolysis

All cultures of Arthrobacter showed hydrolysis of casein when they were grown on milk agar. Clearing of the milk agar usually extended to more than 1 cm. beyond the edge of the growth in giant colony streaks incubated 7 days at 28° C.

None of the 5 cultures of the Jensenia-Nocardia group showed hydrolysis of casein on milk agar plates. Growth was secured, and it was abundant for cultures 27, 28, and 31. All the cultures of Microbacterium also failed to hydrolze casein.

Among the animal diphtheroids, only cultures 42 and 43 produced clearing on milk agar. Satisfactory growth was obtained for cultures 32, 33, 34, 36, 37, 38, 39, 40, 41, and 44, but none of these caused any clearing in the substrate. Among the cellulolytic and phytopathogenic corynebacteria, there also occurred a few cultures which hydrolyzed casein. These were cultures 51 and 55. The remaining cultures of groups IV and V failed show hydrolysis of casein.

Urea, uric acid, and sodium hippurate hydrolysis

Sixteen of the 60 cultures produced urease. Urease production was not noted for any culture of the phytopathogenic or cellulolytic corynebacteria, nor for any culture of Microbacterium. Eight cultures (4, 5, 6, 8, 10, 22, 23, 25) of the Arthrobacter group, cultures 27 and 28 in the Jensenia-Nocardia group, and cultures 33-37 and 45 of the animal corynebacteria produced urease.

Several cultures of Arthrobacter, and also Cellulomonas fima, all of which gave negative tests for urease production, showed normal amount of growth in four successive subcultures on a medium containing urea as the only added source of nitrogen.

Thirteen of the 60 cultures hydrolyzed uric acid. All of the 8 cultures of the Arthrobacter group listed above as positive for urease production also were capable of hydrolyzing uric acid. In addition 4 cultures of Arthrobacter negative for urease production gave positive tests for uric acid hydrolysis. These were Arthrobacter aurescens, cultures 1, 2, and 3, and the unnamed culture 21.

The only culture outside the Arthrobacter group that was capable of hydrolyzing uric acid was culture 42, an unnamed animal diphtheroid.

All of the 60 cultures listed in Table 1 gave negative responses when tested for hydrolysis of sodium hippurate.

Nitrate reduction

The nitrate medium on which subcultures were grown appeared important in determining whether a positive or a negative test for nitrate reduction was obtained. On plain nitrate agar as employed by Dimmick (25), 33 of the 60 cultures demonstrated ability to reduce nitrates to nitrites. On a peptone-enriched form of Dimmick's medium, 30 of the 60 cultures were found to reduce nitrates. But only 22 of the 60 cultures gave positive tests on both plain and enriched nitrate agar.

Some cultures were unable to grow on plain nitrate agar and therefore if capable of reducing nitrates were among those that accomplished reduction on the enriched agar. Other cultures grew equally well on both media, and yet some of them reduced nitrates only on the plain agar, and others, only on the enriched agar.

A summary of observations concerning nitrate reduction is presented in Table 3.

Production of ammonia, indole, and hydrogen sulfide

None of the 60 cultures produced ammonia in peptone broth subcultures that had been incubated 4 and 10 days prior to the addition of Nessler's solution as the test reagent. Neither were any of the cultures found capable of producing indole.

In the Arthrobacter group, a few cultures were encountered which produced hydrogen sulfide. Arthrobacter tumescens 11 and 12, A. helvolum 10, A. simplum 8, and Corynebacterium simplex produced browning or bleckening of the test medium after 14 to 18 days of incubation. The remaining cultures of group I gave negative responses.

Of the cultures listed under groups II through VI, culture 45, an unnamed animal diphtheroid, showed early blackening of the test medium. Cultures 52 and 53 showed some amber to brownish discoloration after two weeks of incubation.

Table 3. Nitrate Reduction and Carbohydrate Fermentation Responses

Culture No.	Nitrate reduction on:		Carbohydrate fermentation responses									
	Dextrose-nitrate agar	Dextrose-nitrate-peptone agar	Xylose	Arabinose	Dextrose	Galactose	Maltose	Sucrose	Lactose	Glycerol	Mannitol	Inositol
Group I.	Arthrobacter:											
1.	+	+	+	+	+	+	+	+	+	+	+	+
2.	+	+	+	+	+	+	+	+	+	+	+	+
3.	+	-	+	+	+	+	+	+	+	+	+	+
4.	+	-	+	+	+	+	+	+	+	+	+	+
5.	+	+	+	+	+	+	+	+	+	+	+	+
6.	+	+	+	+	+	+	+	+	+	+	+	+
7.	+	+	-	-	+	+	+	+	-	+	+	+
8.	+	+	+	+	+	+	+	+	+	+	+	+
9.	+	-										
10.	+	-	+	+	+	+	+	+	+	+	+	+
11.	-	+	-	-	-	-	-	-	-	-	-	-
12.	-	+	-	-	-	-	-	-	-	-	-	-
13.	+	+	-	-	+	+	-	+	-	-	-	-
14.	+	-	-	-	+	+	+	+	+	+	+	+
15.	+	-	-	-	+	-	-	+	-	+	-	-
16.	+	+	-	-	-	-	-	-	-	-	-	-
17.	-	-	-	-	-	-	-	+	-	+	-	-
18.	-	-										
19.	+	-	-	-	+	+	+	+	-	+	+	+
20.	+	+	-	-	+	+	-	+	-	+	+	-
21.	+	+	-	-	+	+	-	+	-	+	-	-
22.	+	+	+	+	+	+	+	+	+	+	+	+
23.	+	+	+	+	+	+	+	+	+	+	+	+
24.	+	-	+	+	+	+	+	+	+	+	+	+
25.	+	+	-	-	+	+	+	+	+	+	-	-
26.	+	-	+	+	+	+	+	+	+	+	+	+

Table 3 (continued)

Culture No.	Nitrate reduction on:		Carbohydrate fermentation responses									
	Dextrose-nitrate agar	Dextrose-nitrate-peptone agar	Xylose	Arabinose	Dextrose	Galactose	Maltose	Sucrose	Lactose	Glycerol	Mannitol	Inositol
Group II. Jensenia-Nocardia:												
27.	+	+	-	+	+	+	-	+	-	+	+	+
28.	+	+	+	+	+	+	+	+	+	+	+	+
29.	-	-	-	-	-	-	-	-	-	-	-	-
30.	-	-	-	-	-	-	+	-	-	+	-	-
31.	-	+	+	+	+	+	+	+	+	+	+	+
Group III. Animal diphtheroids:												
32.	-	-	+	+	+	+	+	+	+	+	+	+
33.	-	-	+	+	+	+	+	+	+	+	+	+
34.	-	+	-	-	+	-	-	-	-	-	-	-
35.	-	-	-	-	-	-	-	-	-	-	-	-
36.	-	+	-	-	+	-	-	+	-	-	-	+
37.	-	+	-	-	+	-	+	+	-	-	-	+
38.	-	+	-	-	+	-	-	-	-	-	-	-
39.	+	+	-	-	+	-	-	-	-	-	-	-
40.	-	-	-	-	+	-	-	-	-	-	-	-
41.	-	-	-	-	+	-	-	-	-	-	-	-
42.	+	+	+	+	+	+	+	+	+	+	+	+
43.	+	+	-	-	-	-	-	-	-	-	-	-
44.	-	+	-	-	+	-	-	-	-	-	-	-
45.	-	-	-	-	-	-	-	-	-	-	-	-
Group IV. Cellulolytic corynebacteria:												
46.	-	-	+	+	+	+	-	+	+	+	-	-
47.	+	-	-	-	/	-	-	-	-	-	-	-
48.	-	-	+	+	+	+	+	+	+	+	-	-
49.	+	+	-	-	+	+	+	+	+	-	-	-
50.	+	-	-	-	+	-	-	-	-	+	+	-

Table 3 (continued)

Culture No.	Nitrate reduction on:		Carbohydrate fermentation responses									
	Dextrose-nitrate agar	Dextrose-nitrate-peptone agar	Xylose	Arabinose	Dextrose	Galactose	Maltose	Sucrose	Lactose	Glycerol	Mannitol	Inositol
51.	+	+	-	-	+	+	+	+	+	-	-	-
52.	-	-	-	-	+	+	+	+	+	-	-	-
53.	+	+	-	-	+	+	+	+	+	-	-	-
54.	+	+	-	-	+	-	-	-	-	-	-	-
Group V.	Phytopathogenic corynebacteria:											
55.	-	-	+	+	+	+	+	+	+	+	-	-
56.	-	-	-	-	+	-	+	+	-	-	-	-
57.	-	-	-	-	+	+	+	+	-	+	-	-
Group VI.	Microbacteria:											
58.	-	-	-	-	+	+	+	+	+	+	-	-
59.	-	-	-	-	+	+	+	-	+	-	-	-
60.	-	-	-	-	+	+	-	-	+	-	-	-

Production of catalase on nutrient agar

All cultures gave positive responses for catalase production when tested with hydrogen peroxide.

Production of acetylemethylcarbinol and of acidity in dextrose broth

None of the 60 cultures employed for study produced acetylmethylcarbinol in dextrose broth.

There were differences among the cultures in their ability to produce acidity in dextrose broth. None of the Arthrobacter group of cultures increased the acidity of nutrient broth beyond the reaction initially present. In broth initially adjusted to pH 6.8, cultures 1-26, inclusive produced pH reactions ranging from 6.8 to 7.2 after 4 days of incubation at room temperature. With additional 6 days of incubation pH reactions of the individual cultures became slightly more alkaline.

Jensenia canicruria, 27, cultures 28-30, and Nocardia rubra 31 produced neutral to alkaline reactions in dextrose nutrient broth. Their pH values were determined as 7.5, 7.5, 6.9, 7.0, and 6.9, respectively, after 4 days of incubation.

Nearly all of the animal diphtheroids produced acidity in glucose broth. The exceptions were cultures 32, 33, and 43, which produced reactions of 7.6, 7.4, and 7.7, respectively.

All of the cellulolytic corynebacteria produced acidity in dextrose nutrient broth. Cultures 46-54 inclusive yielded pH values of 5.3, 5.3, 5.0, 4.7, 5.4, 5.5, 6.2, 6.0, and 5.7, respectively. The

cultures of Microbacterium also produced early acidity, and pH values of 4.8 to 5.0 were observed. The phytopathogenic corynebacteria failed to produce early acidity, after 4 days of incubation, pH values for cultures 55, 56, and 57 were determined 7.0, 6.8, and 6.8 respectively. After an additional 10 days, pH values determined were 6.8, 6.7 and 6.7.

Utilization of citrate

Cultures 11 and 12 of Arthrobacter tumescens and culture 17 of Bacterium globiforme failed to grow on sodium citrate medium. The remaining 23 cultures of the Arthrobacter group produced growth and alkalinity within 2 days.

Jensenia canicruria 27, culture 28, and Nocardia rubra 31 were citrate positive; cultures 29 and 30 of group II were citrate negative.

All cultures of groups IV, V, and VI, that is, of the cellulolytic and phytopathogenic corynebacteria and of Microbacterium, failed to show utilization of citrate.

Hydrolysis of starch

All cultures of Arthrobacter group, after growth for 7 days on starch nutrient agar, showed hydrolysis of starch on plates developed with alcohol. Zones of 2.5 cm. or more in width were secured for all cultures except for 6, 7, 8, and 9 strains of Arthrobacter simplum, which produced clearing that was almost entirely under the line of

growth and from 0.5 to 1 cm. in width.

Duplicate plates of all Arthrobacter cultures, again excepting only the cultures of A. simplum referred to above, gave positive responses when tested for starch hydrolysis by addition of Lugol's iodine solution. Although no clear zones were immediately apparent upon addition of the iodine solution to nutrient starch plates of A. simplum, it was observed that when such plates had been allowed to stand at room temperature for 1 hour, the blue to purple color under the line of streak began to fade, and continued to fade beyond the streak until a zone comparable to that given by the alcohol test method had become cleared. When A. simplum was incubated for 21 days prior to addition of iodine, a clear zone immediately became apparent.

Among the Jensenia-Nocardia group II, cultures 27, 28, and 31 were negative for starch hydrolysis at 7 days, but 27 and 28 were weakly positive for 21 days. Cultures 29 and 30 showed hydrolysis of starch when tested with alcohol after 7 days of incubation.

All cultures of the phytopathogenic and cellulolytic corynebacteria and of microbacteria (groups IV, V, VI) showed hydrolysis of starch after 7 days of incubation and with either alcohol or Lugol's iodine as the testing solution.

Rather erratic results were noted among the fourteen corynebacteria of animal origin. Seven cultures, (32, 33, 35, 37, 40, 41, 43) showed no diastase action, cultures 34, 36, 38, 39 showed weak action, and cultures 42, 44, 45 showed distinctly positive starch hydrolysis.

Fermentation responses

The fermentation responses of individual cultures frequently failed to show agreement in separately conducted tests. Whether these failures were caused by variations in differing lots of the test media or by differences in the inocula employed was not determined. Acid production when present was usually limited to the slanted portion of the agar.

Within each of the six groups, there were some cultures capable of producing acid from nearly all of the test carbohydrates, and there were others which produced acid from few or none of these carbohydrates. The responses of individual cultures on ten carbohydrate-containing media are shown in Table 3.

DISCUSSION

The common occurrence both of branching cells in young subcultures and also of elongated cells which with aging became fragmented into shorter units supports the point of view that the soil globiforme bacteria belong in the order Actinomycetales and not in the Eubacteriales. The occurrence of smooth colonies and of early fragmenting mycelia in the proactinomycetes has been described by Umbreit (57) and McClung (44). The cell pattern in young colonies of the corynebacteria of soil, plant, or animal origin suggests a close relationship between these bacteria and the proactinomycetes. Further evidence of such a relationship is shown by the common occurrence of lateral budding and branching in the corynebacteria.

The statment of Bisset and Moore (9) that the corynebacteria show a compound cell structure has to some extent been confirmed in the present study. Nevertheless their general conclusions do not appear to be valid. Observations both on unstained cells in situ on thin agar films and on cell smears stained by a tannic acid-crystal violet method have suggested that the multi-celled units of Bisset and Moore (9) are transitory, and appear most commonly at the time elongated, young cells undergo fragmentation into short rods and coccoids. Fragmentation of this type has been fully described by McClung (44) for proactinomycetes which form an early or transitory mycelium. In such organisms, formation of cross walls in the initially long and multi-branched mycelial cells is followed by fragmentation

and the appearance of a number of short cell units. In the corynebacteria, the pattern is similar, except that the young cells do not form an initial early mycelium. Upon attainment of cell lengths equal to 4 to 8 diameters, the individual cells become subdivided by cross walls, and the "compound" cells of Bisset and Moore (9) can be demonstrated. Fragmentation of these compound cells results in the replacement of the young or rod stage of growth of the globiforme group with the mature or coccoid type of cells. This pleomorphism was fully described by Conn (18) in his initial description of Bacterium globiforme.

The statements of Bisset (8) that Jensenia canicruria is representative of the soil diphtheroids, that his species is "single-celled" in contrast to the corynebacteria proper, and that the deeply staining granules commonly seen in stained cells of the corynebacteria are artifacts of the staining procedures employed all appear to be in error. All the soil diphtheroids or soil globiforme bacteria herein studied which initially were isolated by Jensen, Conn, or Lochhead were culturally dissimilar from Jensenia canicruria andin addition failed to form an early open mycelium. Only culture 28 in the current collection was found identical to J. canicruria; culture 28 was isolated initially by Topping (56), and it was described by her as a mycelium-forming organism which very probably belonged to the proactinomycetes. Bisset and Moore (9) failed to obtain named cultures from Conn and others, and apparently they failed to recognize the close relationship of their proposed genus Jensenia to

Nocardia. Mycelium formation by Jensenia, and its failure to hydrolyze casein or to show diastatic action on starch suffice to show that Jensenia is not in synonymy with Arthrobacter.

Cytological studies employing both light and electron microscopy have shown that Jensenia canicruria is not single-celled at all times (see Fig. 15). It is the writer's opinion that the morphological distinctions made by Bisset and Moore (9) are unsound, and that the differences reported are no greater than those that might be encountered in examining differing growth stages of the same subculture. Electron microscopy also has revealed the occurrence of electron-opaque bodies in unstained cells of corynebacteria from soil, plant and animal sources. Any conclusion that the metachromatic granules of corynebacteria are artifacts of staining therefore appears to be unwarranted.

The soil corynebacteria isolated by Conn, Jensen, Topping, and Lochhead, and those isolated locally, have been found to comprise a homogeneous group. This group is distinguished from corynebacteria of animal origin primarily by its greater proteolytic activity, its ability generally to use inorganic sources of nitrogen, its inability to produce acidity in dextrose nutrient broth, and its failure to grow at 37° C. In view of such differences, it does not appear advisable to include this group of soil bacteria in the genus whose type is Corynebacterium diphtheriae.

If Corynebacterium is rejected as the generic assignment for the soil globiforme bacteria, a question then arises whether there

does exist any suitable and valid genus to which the soil globiforme bacteria properly can be assigned. Conn and Dimmick (21) have proposed that the soil corynebacteria be placed in Arthrobacter. This genus can be accepted only if cellulolytic corynebacteria are recognized as generically distinct. If they are not so recognized, the genus Cellulomonas has priority over Arthrobacter. There appears sufficient justification for the generic separation of the cellulolytic bacteria of Kellerman et al. (33) and the Arthrobacter group of Conn and Dimmick (21). The cellulolytic activity of Cellulomonas, the ability of this genus to produce distinct acidity in dextrose nutrient broth, and the inability of all cultures in this group to hydrolyze urea or uric acid distinguish this genus from Arthrobacter.

The phytopathogenic corynebacteria and the majority of the animal diphtheroids appeared to occupy a position intermediate between Arthrobacter and Cellulomonas. Acid production was variable. Organic sources of nitrogen or growth factors were generally required in order that satisfactory growth could be obtained. One of the animal diphtheroids, unnamed culture 42, possessed the general characteristics of Arthrobacter and therefore should be designated as member of this genus and not of Corynebacterium. The microbacteria appeared culturally to be related to Cellulomonas, but were distinguished therefrom by their thermal resistance and their inability to attack cellulose. It is very probable that many of the bacteria designated as Microbacterium are incorrectly named, even though such examples were not encountered in this study. Some of the atypical microbacteria,

as well as some of the atypical animal diphtheroids, quite likely would be found to be members of Arthrobacter or even of Cellulomonas if extended studies were undertaken.

Species differentiation within the soil globiforme bacteria has been found difficult by many workers, only two of whom have proposed individual species. Jensen (31), in placing all saprophytic corynebacteria in the genus Corynebacterium, used criteria such as "pronounced morphological differentiation," pigmentation, and growth in protein-free media. Some half dozen species were described. More recently, Conn and Dimmick (19, 21, 22) have proposed recognition of five species of Arthrobacter, namely, A. globiforme, A. helvolum, A simplum, A. tumescens, and A. aurescens. These species were separated on the basis of differences in morphology, diastatic action on starch, growth, and pigmentation. The application of these and of other criteria for species differentiation of the soil globiforme bacteria is not easily accomplished.

Conn and Dimmick (22) described Arthrobacter simplum as a species distinct from A. globiforme primarily because of its failure to attack starch. A. simplum failed to show diastatic action on starch-agar plates tested with Lugol's iodine solution after 7 days of incubation at 30° C. That observation currently has been confirmed. However, when similarly incubated starch-agar plates were tested with alcohol instead of with iodine, diastatic action was revealed. Although the replicate plates treated with iodine immediately became uniformly colored by the added iodine, such plates developed partially cleared

and colorless zones after standing for 30 minutes. These zones corresponded in size to those which developed immediately following addition of alcohol. Also, the diastatic action of A. simplum was apparent on starch-agar plates that had been incubated for 21 days immediately following the addition of iodine. The recognition of A. simplum as a species of Arthrobacter that fails to attack starch does not appear to be justified. No specific differences were encountered between cultures received as A. simplum and cultures received as A. globiforme.

Conn (19) has suggested that the yellow-pigmented varieties of A. globiforme are sufficiently unique that they may well be designated as A. aurescens. The occurrence of deep yellow pigmentation in cultures of A. aurescens has been confirmed. A. helvolum was also noted to produce a yellow pigmentation. Morphological differences among these three species were not apparent. Physiologically, all cultures of A. aurescens herein studied distinguished themselves from other named cultures of Arthrobacter in that all gave negative tests for urease while positive for uric acid hydrolysis. Other named cultures of Arthrobacter were uric acid negative, or if positive, they were also positive when tested for urease production.

No physiological differences were noted between Arthrobacter globiforme and A. helvolum. The greater morphological irregularity of A. helvolum reported by Conn and Dimmick (21) appeared insufficient for species separation.

Conn and Dimmick (21) recognized Arthrobacter tumescens as a species which failed to show branching of cells, lacked diastatic action on starch, and failed to grow on protein-free media. In the current work, both branching of cells and production of diastase by A. tumescens have been noted. The species failed to grow on protein-free media. Several cultures of globiforme bacteria initially isolated by Lochhead or by Topping also failed to grow on protein-free media. A. tumescens apparently resembles the type II of Bacterium globiforme described by Taylor (54). Taylor frequently encountered his type II bacteria in Canadian soils. Inasmuch as this type is reported as abundant in soil, it probably is best to retain A. tumescens as a species distinct from A. globiforme.

Carbohydrate fermentation responses were of no value in species differentiation. Taylor (54) summarized his work on the availability of different carbon compounds to the globiforme group with the statement that the inconsistent and changeable reactions produced on different sugars only emphasized the futility of any attempt to classify the organisms on the basis of acid production.

The application of diverse tests to a considerable number of saprophytic corynebacteria has revealed a general uniformity in many of their responses. All of the saprophytic corynebacteria lacked the thermal resistance exhibited by the microbacteria. All the corynebacteria failed to produce ammonia or indol in peptone broth subcultures. All failed to hydrolyze sodium hippurate, and none of them produced acetylmethylcarbinol in dextrose nutrient broth. All of them gave

positive responses for catalase production when tested with hydrogen peroxide. Demonstration of such uniformity has helped to characterize more fully the soil globiforme bacteria.

SUMMARY

In order to characterize more fully the soil globiforme bacteria, morphological and physiological studies were made both on representative cultures of this group and on various other bacteria whose generic relationships to the soil globiforme bacteria remain poorly defined.

By direct microscopy with the light microscope, motility was noted for 10 of 26 cultures of soil globiforme bacteria, and cell branching, for all of them. Examination of cells in micro-colonies on thin agar films showed that Jensenia canicruria formed a distinct early mycelium. The soil globiforme, animal diphtheroid, cellylolytic, and phytopathogenic bacteria, and microbacteria examined failed to show noteworthy differences in cell size or shape, or in their micro-colonies. Fragmentation within micro-colonies was sufficiently rapid to prevent formation of an early open mycelium.

Observations made on cell smears stained by a tannic acid-crystal violet method failed to confirm recent claims that the soil globiforme bacteria possess simple cells, while the true corynebacteria possess compound cells. Cross-walled or compound cells were demonstrated in Jensenia canicruria as well as in Arthrobacter globiforme. Jensenia was found dissimilar, physiologically and in early mycelium formation, from the majority of named cultures designated as representative of the soil globiforme bacteria. It was concluded that Jensenia is not in synonymy with Arthrobacter, but that it very probably is in synonymy with Nocardia.

Except for Jensenia, the soil corynebacteria isolated by widely scattered workers were found to comprise a homogeneous group. This group was distinguished from corynebacteria of animal origin by its greater proteolytic activity, its ability generally to grow on protein-free media, and its inability to produce acidity in dextrose nutrient broth. In view of these and other differences, it does not appear advisable to include the soil globiforme bacteria in the genus whose type is Corynebacterium diphtheriae. It was concluded that Arthrobacter constitutes a valid generic designation for the soil globiforme bacteria.

Arthrobacter cultures were compared to cultures of Cellulomonas, phytopathogenic corynebacteria, animal diphtheroids, and microbacteria in numerous cultural and physiological tests. All cultures were uniformly negative with respect to ammonia, indol, and acetylmethylcarbinol production and hydrolysis of sodium hippurate; all were uniformly positive for catalase production. Some differences were encountered in gelatinolysis, urea and uric acid hydrolysis, nitrate reduction, action on various organic carbon compounds, and in character of growth on diverse media. Carbohydrate fermentation responses were of no value in group or in species differentiation.

Contrary to its type description, Arthrobacter simplum was found to show diastatic action on starch-agar plates. The recognition of A. simplum as a species of Arthrobacter that failes to attack starch does not appear to be justified. Both A. aurescens and A. helvolum produced deep yellow pigmentation on nutrient agar and on potato, and

both produced hydrolysis of uric acid. A. aurescens was urease negative, and A. helvolum, urease positive. A. globiforme and A. tumescens failed to produce deep yellow pigmentation. A. tumescens, described recently as lacking diastatic action and as producing no branching of cells, was found diastase positive and capable of producing branching cells in young subcultures. A. tumescens failed to grow on protein-free media; it showed marked caseinolysis on milk agar. A. tumescens probably should be retained as a species of Arthrobacter in addition to A. globiforme.

LITERATURE CITED

1. Allen, O. N. Experiments in soil bacteriology. Minneapolis, Burgess Publishing Co. 1949.

2. Ayers, S. H. and Rupp, P. Differentiation of hemolytic streptococci from human and bovine sources by the hydrolysis of sodium hippurate. J. Infect. Dis. 30:388-399. 1922.

3. Bergey, D. H. and others. Bergey's manual of determinative bacteriology. 1st ed. Baltimore, the Williams and Wilkins Co. 1923.

4. _____ and _____ Bergey's manual of determinative bacteriology. 2nd ed. Baltimore, the Williams and Wilkins Co. 1925.

5. _____ and _____ Bergey's manual of determinative bacteriology. 3rd ed. Baltimore, the Williams and Wilkins Co. 1930.

6. _____ and _____ Bergey's manual of determinative bacteriology. 4th ed. Baltimore, the Williams and Wilkins Co. 1934.

7. _____ and _____ Bergey's manual of determinative bacteriology. 5th ed. Baltimore, the Williams and Wilkins Co. 1939.

8. Bisset, K. A. The cytology and life-history of bacteria. Edinburgh, E. & S. Livingstone, Ltd. 1950.

9. _____ and Moore, F. W. The relationship of certain branched bacterial genera. J. Gen. Microbiol. 3:387-391. 1949.

10. _____ and _____ Jensenia, a new genus of the Actinomycetales. J. Gen. Microbiol. 4:280. 1950.

11. Breed, R. S., Murray, E. G. D., and Hitchens, A. P. Bergey's manual of determinative bacteriology. 6th ed. Baltimore, the Williams and Wilkins Co. 1948.

12. Clark, Francis E. Notes on types of bacteria associated with plant roots. Trans. Kansas Acad. Sci. 43:75-84. 1940.

13. _____ The generic classification of certain cellulolytic bacteria. Soil Sci. Soc. Amer. Proc. 15:180-182. 1951.

14. _____ and Carr, P. H. Motility and flagellation of the soil corynebacteria. J. Bact. 62:1-6. 1951.

15. Committee on Bacteriological Technique. Manual of methods for pure culture study of bacteria. Soc. of Am. Bact. Geneva, N. Y., Biotech. Publications. 1946.

16. Conn, H. J. Soil flora studies. IV. Non-spore-forming bacteria in soil. N. Y. State Agri. Exp. Sta. Tech. Bul. 59. 1917.

17. _____ Soil flora studies. VI. The punctiform-colony forming bacteria in soil. N. Y. State Agri. Exp. Sta. Tech. Bul. 115. 1925.

18. _____ A type of bacteria abundant in productive soils, but apparently lacking in certain soils of low productivity. N. Y. State Agri. Exp. Sta. Tech. Bul. 138. 1928.

19. _____ Geneva, N. Y. Remarks on Arthrobacter aurescens. (Private communication to F. E. Clark) 1950.

20. _____ and Darrow, M. A. Characteristics of certain bacteria belonging to the autochthonous microflora of soil. Soil Sci. 39:95-110. 1935.

21. _____ and Dimmick, Isabel Soil bacteria similar in morphology to Mycobacterium and Corynebacterium. J. Bact. 54:291-303. 1947.

22. _____ and _____ Soil bacteria of the genus Arthrobacter. Soil Sci. 65:349-351. 1948.

23. _____ Wolfe, G. E., and Ford, M. Taxonomic relationships of Alcaligenes spp. to certain soil saprophytes and plant parasites. J. Bact. 39:207-226. 1940.

24. Cowan, S. T. Ideas for an outline classification of the schizomycetes. 5th Internatl. Congr. Microbiology. Rio de Janeiro, August, 1950. (Mimeo. rept.).

25. Dimmick, Isabel Phosphorus deficiency in relation to nitrate reduction test. Can. J. Res. Sect. C. 25:271-273. 1947.

26. Dowson, W. J. On the generic name of the gram-positive bacterial plant pathogens. Brit. Myc. Soc. Trans. 25:311-314. 1942.

27. _____ Manual of Bacterial plant diseases. London. Adam and Chas. Black. 1949.

28. Fischer, Alfred Utersuchungen uber Bakterien. Jahrb. Wiss. Botan. 27:1-163. 1895.

29. Janke, Von Alexander. Zur Bakterien-systematik. Cent. f. Bakt. II Abt. 80:481-482. 1930.

30. Jensen, H. L. Corynebacteria as an important group of soil microorganisms. Linn. Soc. N. S. Wales, Proc. 58:181-185. 1933.

31. _____ Studies on saprophytic mycobacteria and corynebacteria. Linn. Soc. N. S. Wales, Proc. 59:19-61. 1934.

32. Kellerman, K. F. and McBeth, I. G. The fermentation of cellulose. Cent. f. Bakt. Abt. II. 34:485-499. 1912.

33. _____ and others Identification and classification of cellulose-dissolving bacteria. Cent. f. Bakt. II Abt. 39:502-522. 1913.

34. Kluyver, A. J. and Van Niel, C. B. Prospects for a natural system of classification of bacteria. Cent. f. Bakt. II Abt. 94:369-403. 1936.

35. Krassilnikov, N. A. Die Entwicklungsgeschichte der Bodenmykoloakterien. Cent. f. Bakt. II Abt. 90:428-434. 1934.

36. Lehmann, K. B. and Neumann, R. O. Atlas und Grundriss der Bakteriologischen und Lehrbuch der speciellen Bakteriologischer diagnostik. 1 aufl. München, J. F. Lehmann. 1896.

37. _____ and _____ Atlas and principles of bacteriology and text book of special bacteriologic diagnosis. Translation from the second enlarged and revised German edition. Edited by G. H. Weaver. Part II-Text. Philadelphia, W. B. Sanders Co. 389-409. 1901.

38. _____ and _____ Atlas und Grundriss der Bakteriologie und Lehrbuch der speziellen Bakteriologischen. 4 aufl. München, J. F. Lehmann. 1907.

39. _____ and _____ Bakteriologische, isbesondere Bakteriologische diagnostik. 7 aufl. München, J. F. Lehmann. 1926-27.

40. Lochhead, A. G. Quantative studies of soil microorganisms. III. Influence of plant growth on the character of bacterial flora. Can. J. Res. Sect. C. 18:42-53. 1940.

41. _____ Chromogenic bacteria related to Bacterium globiforme. J. Bact. 55:579-580. 1948.

42. McBeth, I. G. Studies on the decomposition of cellulose in soils. Soil Sci. 1:437-487. 1916.

43. _____ and Scales, F. M. The destruction of cellulose by bacteria and filamentous fungi. U.S.D.A., B.P.I. Bul. 266. 1913.

44. McClung, N. M. Morphological studies in the genus Nocardia. I. Developmental studies. Lloydia 12:137-177. 1949.

45. Nashif, Selim A. Microbacteria from Iowa dairy products. Unpublished M.S. Thesis. Ames, Iowa, Iowa State College Library. 1949.

46. Pribram, Ernst. A contribution to the classification of micro-organisms. J. Bact. 18:361-394. 1929.

47. Pringsheim, E. G. Zur Kritik der Bakteriensystematik. Lotos. 71:357-377. 1923.

48. Robinow, C. F. Addendum to the bacterial cell by R. J. Dubos. Cambridge, Mass., Harvard University Press. 1945.

49. Schaub, I. G. and Foley, M. K. Diagnostic bacteriology. 3rd ed. St. Louis, The C. V. Mosby Co. 1947.

50. Skaptason, J. B. and Burkholder, W. H. Classification and nominclature of the pathogen causing bacterial ring rot of potatoes. Phytopath. 32:439-441. 1942.

51. Smith, N. R., Gordon, Ruth E., and Clark, F. E. Aerobic mesophilic spore-forming bacteria. U.S.D.A. Mics. Pub. 559. 1946.

52. Stanier, R. Y. and Van Niel, C. B. The main outlines of bacterial classification. J. Bact. 42:437-466. 1941.

53. Starr, M. P. and Pirone, P. P. Phytomonas poinsettiae n. pp., the cause of a bacterial disease of poinsettia. Phytopath. 32:1076-1081. 1942.

54. Taylor, C. B. Further studies of Bacterium globiforme and the incidence of this type of organism in Canadian soils. Soil Sci. 46:307-321. 1938.

55. _____ and Lochhead, A. G. A study of Bacterium globiforme Conn in soils differing in fertility. Can. J. Res. Sect. C. 15:340-347. 1937.

56. Topping, Lucy E. The predominant micro-organisms in soils. I. Description and classification of the organisms. Cent. f. Bakt. II Abt. 97:289-304. 1937.

57. Umbreit, W. W. Studies on the proactinomyces. J. Bact. 38: 73-89. 1939.

58. Waksman, S. A. Principles of soil microbiology. 2nd ed. Baltimore, the Williams and Wilkins Co. 1932.

59. Wallace, G. I. and Neave, S. L. The nitrite test as applied to bacterial cultures. J. Bact. 14:377-384. 1927.

60. Winslow, C. E. A. and others. The families and genera of bacteria. J. Bact. 2:505-566. 1917.

ACKNOWLEDGMENT

The author wishes to express his sincere appreciation and deep gratitude to Dr. Francis E. Clark for suggestions in planning and carrying out the research and in the preparation of the manuscript. He is also indebted to Dr. P. H. Carr for making the electron micrographs.